Risk assessment of metritis cure for antibiotic-treated and not-treated dairy cows: designing a decision tree for selective treatment

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# Risk assessment of metritis cure for antibiotic-treated and not-treated dairy cows: designing a decision tree for selective treatment

By

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To my wife Tania and my dad Ribamar for all the support and encouragement given to me all my life.

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| | |
|---|---|
| AIC | Akaike information criteria |
| AMR | Antimicrobial resistance |
| AUC | Area under the curve |
| BCS5 | Body condition score at day five post-partum |
| BCSC | Body condition score at calving |
| BHB | Beta-hydroxybutyrate |
| CEF | Ceftiofur crystalline-free acid |
| CON | No treatment at the time of metritis diagnosis |
| DAMPs | Damage-associated molecules or molecular patterns |
| DIM | Days in milk |
| DIM D0 | Days in milk at metritis diagnosis |
| FDA | Food and Drug Administration |
| FDR | False discovery rate |
| FN | False negatives |
| FP | False positives |
| FS | Feature scaling |
| GC-TOF-MS | Untargeted gas chromatography time-of-flight mass spectrometry |
| KNN | K-Nearest neighbour |
| LOF | Local outlier factor |
| MET | Cows with metritis |
| MilkVar | Milk production from the day before to the metritis diagnosis categoric |
| ML | Machine learning |
| MS | Mass spectrometry |

| | |
|---|---|
| MVI | Missing value imputation |
| NEFA | Non-esterified fatty acids |
| NMR | Nuclear magnetic resonance |
| NoMET | Counterparts without metritis |
| NPV | Negative predictive value |
| OD | Outlier detection |
| P | Percentile |
| PLS-DA | Partial least squares discriminant analysis |
| PPV | Positive predictive value |
| REML | Restricted maximum likelihood |
| RFE | Recursive feature elimination |
| RFM | Retained fetal membranes |
| ROC | Receiver operating characteristic |
| RSPL | Residual subject-specific pseudo likelihood |
| Se | Sensitivity |
| SMC | Spontaneous metritis cure |
| Sp | Specificity |
| TMR | Total mixed ratio |
| TN | True negative |
| TP | True positive |
| VLC | Categorized vulvovaginal laceration |
| VLS | Vulvovaginal laceration score |
| Vmilk | Milk production from the day before to the metritis diagnosis continuous |
| WHO | World health organization |

# INTRODUCTION

Metritis, a polymicrobial acute inflammatory disease of the uterus, affects 20 to 40% of dairy cows in the first 21 days postpartum (Pinedo et al., 2020; Galvao, 2012). Metritis is associated with severe clinical signs, increased mortality, impaired wellness, decreased milk production, and fertility, with estimated annual losses of ~1 billion in the US (Silva et al., 2021). Metritis diagnosis is based on a fetid, watery, red-brown discharge, concurrent or not with fever, inappetence, dehydration, and prostration (Sheldon et al., 2019). Recent reports revealed that diagnosis of metritis is widely inconsistent in commercial dairies leading to under and overdiagnosis (Espadamala et al., 2018). Furthermore, due to the high prevalence of this disease, within the dairy industry, the treatment of metritis is one of the major drivers of antimicrobial drug usage in lactating cows (Kaniyamattam et al., 2020).

Ceftiofur, a third-generation cephalosporin that belongs to medically important antimicrobial, is the most common antibiotic used for metritis worldwide (Espadamala et al., 2018). The World Health Organization has warned of the critical need to improve the judicious use of drugs such as ceftiofur to help curtail antimicrobial resistance (AMR) epidemics (FDA 2020). Metritis has been recognized as a disease that offers an opportunity to assess strategies to reduce AMR in farm animals. Studies comparing the cure-risk of treated and non-treated metritic cows revealed that more than half (55-62%) of metritis cases undergo spontaneous cure, while the cure risk of cows treated with ceftiofur ranges from 75 to 79% (de Oliveira et al., 2020; McLaughlin et al., 2012; Chenault et al., 2004). Albeit it is known that antimicrobial therapy changes the outcomes of clinical cure in only 20% of metritic cows, we cannot predict who those cows are.

Our overall objective is to develop a novel algorithm approach to identify animals that spontaneously cure from metritis, reducing antibiotic usage in dairy farm. Taking into consideration the high prevalence of uterine diseases in dairy farms and the dairy cow population in the US (9 million cows), approximately 2 million cows receive antimicrobial therapy for the treatment of metritis each year. If successful, the use of the algorithm can improve the judicious use of antibiotics in dairy farms. Minimizing the use of antimicrobials in food-producing animals is expected to reduce the number of residue violations in milk and meat and reduce the risk of increased antimicrobial resistance of certain classes of drugs, such as the third generation of cephalosporin that can share common AMR with similar drugs used in human medicine. With the increased concern about antibiotic use in food-producing animals, in particular, the use of ceftiofur, a decision tree for selective treatment is expected to garner the support of the dairy industry and the general public. Overall, the development of tools to more accurately predict spontaneous cure will support animal health and welfare and help secure the future of the dairy industry. Our goal is to understand what factors may contribute to metritis development, severity, and cure and to develop a selective treatment for metritis optimizing animal welfare, judicious use of antimicrobials in dairy farming, and profitability.

## CHAPTER 1

# INTEGRATION OF STATISTICAL INFERENCES AND MACHINE LEARNING ALGORITHMS FOR PREDICTION OF METRITIS CURE IN DAIRY COWS

## Abstract

The study's objectives were to identify cow-level and environmental factors associated with metritis cure, to predict metritis cure using traditional statistics and machine learning algorithms. The dataset used is from a previous study comparing the efficacy of different therapies and self-cure for metritis. Metritis was defined as fetid, watery, reddish-brownish discharge, with or without fever. Cure was defined as an absence of metritis signs 12 days after diagnosis. Cows were randomly allocated to receive a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the day of diagnosis and three days later (CEF, n = 275); and no treatment at the time of metritis diagnosis (CON, n = 275). The variables days in milk at metritis diagnosis (DIM D0), treatment, season of the metritis diagnosis, month of metritis diagnostic, number of lactation, parity, calving score, dystocia, retained fetal membranes (RFM), body condition score at day five post-partum, vulvovaginal laceration score (VLS), the rectal temperature at the metritis diagnosis, fever at diagnosis, milk production from the day before to metritis diagnosis, and milk production slope up to 5, 7 and 9 DIM were offered to univariate logistic regression. Variables included in the multivariable logistic regression model were selected from the univariate analysis according to P-value ($P < 0.05$). Variables were offered to the model to assess the association between these factors and metritis cure. Additionally, the univariate logistic regression variables were offered to a recursive feature elimination (RFE) to find the optimal subset of features for a machine learning algorithms analysis. Cows without vulvovaginal laceration had 1.91 higher odds of curing of metritis than cows with vulvovaginal laceration. Cows that developed metritis > 7 DIM had 2.09 higher odds of being cured than cows that developed metritis ≤ 7 DIM. For rectal temperature, each degree Celsius above 39.4 ºC leads to a lower odds to be cured than cows with rectal temperature ≤ 39.4 ºC. Furthermore, milk production slope and milk production difference from the day before to the metritis diagnosis were essential variables to predict metritis cure. Cows that had reduced milk production from the day before to the metritis diagnosis had lower odds to be cured than cows with moderate milk production increase. The results from the multivariable logistic regression and ROC analysis indicated that cows developing metritis > 7 DIM, with increase in milk production, and with a rectal temperature ≤ 39.40 oC have increased likelihood of cure of metritis with an accuracy of 75%. The machine learning analysis showed that besides these variables, calving-related disorders, season and month of metritis event are relevant to predict with an accuracy ≥70% and F1-score ≥0.78 whether the cow will cure or not from metritis. Although machine learning algorithms are acknowledged as powerful tools for predictive classification, the current study was unable to replicate its potential benefits. More research is needed to optimize predictive models of metritis cure.

**Keywords:** metritis cure, ceftiofur, dairy cow, machine learning

## Introduction

Metritis, an acute inflammatory disease with systemic implications, affects 20 to 40% of the post-partum dairy cows in the first 21 DIM (LeBlanc et al., 2002; Sheldon et al., 2009; Dubuc et al., 2010). Risk factors such as dystocia, delivery of twin calves, vulva laceration, retained fetal membranes (RFM), or stillbirth can increase the range of metritis cases from 30% to 45% (Markusfeld, 1987; Benzaquen et al., 2007, Vieira-Neto et al., 2016). The consequences of metritis include reduced welfare, increased incidence of other diseases, decreased productive and reproductive performance, increased culling, and decreased profitability (Kossaibati et al., 1997; Sheldon et al., 2004a; Stojkov et al., 2015).

In studies comparing cure-risk of treated and non-treated metritic cows, cows treated with ceftiofur cure-risk ranges from 75 to 78%, while for non-treated cows, the cure-risk ranges from 55 to 62%, a scenario that suggests that approximately two of three cows treated for metritis could recover without antibiotics (Chenault et al., 2004; McLaughlin et al., 2012; de Oliveira et al., 2020). The benefits of using Ceftiofur extend beyond cure rates and include improved productive and reproductive performance and an estimated economic return of $207 per cow (Silva et al., 2021). Third-generation cephalosporin drugs are essential in human and veterinary medicine. The threat of antimicrobial resistance has led the Center for Veterinary Medicine of the Food and Drug Administration to limit certain uses of cephalosporin to preserve antimicrobials' effectiveness for human and veterinary medicine (FDA, 2020).

Reducing traditional antibiotics use is expected to delay the development of bacterial resistance to antibiotics (Fair et al., 2014). Results from a pilot study suggested that not treating metritis cases without fever occurring within the first 5 DIM had no negative outcomes (Sannmann et al., 2013). The development of novel strategies that identify cows with metritis that undergo cure may reduce antibiotic use while also providing economic and sustainability benefits for farmers. Developing models to predict cure based on cow-level and environmental risk factors may help identify cows during the post-partum with metritis that may not need or benefit from the antibiotic.

An alternative approach to develop predictive models in a large dataset such as in agriculture-related studies is machine learning algorithms (Grzesiak et al., 2010). The lack of restrictive parametric assumptions for data analysis and the development of predictive tools makes machine learning algorithms a good candidate for improving the prediction of classic statistical models (Grzesiak et al., 2010). Studies using machine learning have been used to predict cow performance or disease events using large and complex datasets (Shahinfar et al., 2012; Hempstalk et al., 2015). Studies indicated that risk factors for metritis and environmental factors could increase the chance of treatment failure (Machado et al., 2020; de Oliveira et al., 2020). But there is limited information showing if factors influencing metritis cure in treated and non-treated cows are similar. The current study offers a unique platform to evaluate what factors contribute to the success of metritis cure in a randomized experiment with metritic cows receiving or not receiving conventional antimicrobials.

The first objective of this study was to identify cow-level and environmental factors associated with the likelihood of metritis cure. Our second objective was to evaluate the performance of traditional statistical models and a machine learning algorithm to predict metritis cure. Our premise is that cow and environmental-related factors can predict metritis cure with traditional statistical models and machine learning algorithms.

## Materials and Methods

### Data and Animals

Our dataset was from a previous study that compared the efficacy of different therapies and metritis cure (de Oliveira et al., 2020). Briefly, the study was conducted in three dairies located in North Central Florida from May 2016 to June 2017. Cows were diagnosed for metritis at 5, 7, and 9 days in milk (DIM) using the Metricheck® device (Simcro, Hamilton, NZ). Cows with fetid, watery, and reddish-brownish discharge, with or without pieces of necrotic tissue present, were classified as having metritis (Sheldon et al., 2006). Cows diagnosed with metritis were blocked by parity (primiparous or multiparous) and, within each block were randomly assigned to one of three treatments: 1) chitosan microparticles (n = 276) intrauterine infusion of 24 g of chitosan microparticles at diagnosis, two and four days later; 2) subcutaneous injections of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) in the base of the ear at the day of diagnosis and 3 days later (CEF, n = 275); and 3) no treatment at the time of metritis diagnosis (CON, n = 275). Because chitosan microparticles was not an efficacious therapy for metritis, only cows enrolled in the CEF or CON groups were included in the present study (n = 550). At 5 DIM, all cows were scored for body condition (BCS; 1 = thin, 5 = obese; Ferguson et al., 1994) and vulvovaginal laceration (VLS; 0 = no laceration; 1 = laceration < 2 cm at dorsal commissure or internal vaginal wall; 2 = laceration > 2 cm) (Vieira-Neto et al., 2016). The vulvovaginal laceration was categorized (VLC) into No (< 2 cm) or Yes (□ 2 cm). Rectal temperature was measured at enrollment, and cows with a rectal temperature ≥ 39.5 ºC were considered febrile. Additionally, the occurrence of dystocia, twins, RFM, and stillbirth were recorded. The criterion to determine cure was an absence of the characteristic fetid, watery, reddish-brownish discharge at twelve days after enrollment. A cow was considered a treatment failure if she was sold or died before day twelve after metritis diagnosis.

Cows that presented severe dehydration, anorexia, weakness, severe depression, systemic shock, or any other clinical signs attributable to metritis (other than characteristic vaginal discharge and fever) received antibiotic therapy according to the farm protocol and were considered a treatment failure. Milk yield, number of services, average DIM when cows left the herd, reasons for culling, and mortality of the cows enrolled in the study were also recorded. Additional diseases were not accounted in the analysis.

## Statistical Analysis

From the data collected in the study described above, we defined, categorized, and used the following variables for every cow enrolled in the study: DIM D0 (days in milk at metritis diagnosis), categorical days in milk at metritis diagnosis (metritis diagnosed at the first week post-partum vs. diagnosed after the first week post-partum), treatment (CEF vs. CON), season of the metritis diagnosis (cool: September to May vs. hot: June to August), month of metritis diagnosis (January – December), lactation number, parity (primiparous vs. multiparous), calving score (1 = unassisted, 2 = easy pull or slight problem, 3 = moderate pull; and 4 hard pull), dystocia (No: calving score = 1 vs. Yes: calving score > 1), RFM (yes vs. no), body condition score at day five post-partum (BCS5), categorical BCS5 (BCS5C, Low ≤ 3.0, Moderate > 3.0 or < 3.75, or High ≥ 3.75), VLS ((vulvovaginal laceration score) 0, 1 or 2), categorical VLS (VLC, > 2 cm vs. < 2 cm), rectal temperature at the metritis diagnosis, fever at the metritis diagnosis (Yes ≥ 39.5 °C vs. No ≤ 39.5 °C), milk production from the day before to the metritis diagnosis continuous

(Vmilk), milk production from the day before to the metritis diagnosis categoric (MilkVar), and the slope of milk production up to 5, 7 and 9 DIM. The slopes of milk production up to 5, 7, and 9 DIM were calculated using linear regression models (PROC REG, SAS 9.4; SAS Institute Inc., Cary, NC, USA). The model included daily milk production (kg) as a dependent variable and days in milk as an independent variable. Milk increase was categorized based on the percentile (P) of milk production increased obtained from the linear regression (low = P1 – P25; moderate = P25-P75, or high > P75).

Descriptive statistical analysis regarding continuous data, such as DIM D0, the slope of milk production, milk difference, the rectal temperature at enrollment, and BCS at metritis diagnosis, were compared nonparametrically using the PROC NPAR1WAY of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA)., and P-values from the Kruskal-Wallis test are reported. Mean values for normally distributed continuous variables were generated using ANOVA to compare non-cured and cured groups. For the categorical variables (dystocia, parity, RFM, VLC), GLIMMIX procedure of SAS was used to compare cows that cured or failed to cure metritis and PROC FREQ of SAS was used to report proportions.

A three-step approach was used to select the variables to be included in the final multivariate model. First, univariable analyses for each independent variable were performed to assess their association with the odds of metritis cure with the farm as a random effect (de Oliveira et al., 2020), and variables were ranked according to their P-value. Second, multivariable analyses were performed using PROC MIXED and GLIMMIX with maximum likelihood and Laplace parameter estimation methods, respectively. All variables were offered to the model according to their P-value from the univariate analysis (smallest to largest), and the model Akaike Information Criteria (AIC) was used to select the final model variables (stepwise elimination approach). The model with the lowest AIC was used. Finally, PROC MIXED and GLIMMIX were used with restricted maximum likelihood (REML) and residual subject-specific pseudo-likelihood (RSPL) default specifications for parameter estimation. The final model for the multivariate analysis included cure as a dependent variable and the effects of treatment, vulvovaginal laceration, week post-partum of metritis, rectal temperature at metritis diagnosis and milk difference as an independent variable. Furthermore, first-order interactions for all variables were added to the model, and significant ones were kept.

The multivariable analysis results are reported as the ratio of the odds of metritis cure for each risk factor. The unit of analysis was the cow. Statistical significance was declared for all models described above if $P \leq 0.05$, and a tendency was considered if $0.05 < P < 0.10$. For variables that had $P < 0.05$ in the multivariate logistic regression models, receiver operating characteristic (ROC) curves were performed using MedCalc (MedCalc Software, ver. 18.11.6, Mariakerke, Belgium) to determine the most accurate threshold to predict metritis cure. A multivariable ROC curves analysis was performed using the logistic procedure of SAS (PROC LOGISTIC, SAS 9.4; SAS Institute Inc., Cary, NC, USA). The multivariable ROC included the variables that were significant in the multivariable logistic regression model. The results of ROC were reported as sensitivity (Se), specificity (Sp), positive predicted value (PPV), and negative predicted value (NPV) and were considered as follows: less than 0.2 as low, 0.2 to 0.4 as fair, 0.4 to 0.6 as moderate, 0.6 to 0.8 as substantial, and above 0.8 as high (Dohoo et al., 2009).

## Machine Learning Algorithms

**Data preprocessing.** Machine learning algorithms were used to predict cows that would cure or not once diagnosed with metritis. The dataset contained instances (observations) of 550 cows described by 24 attributes (variables), where 22 were independent variables, and 2 were dependent variables (Figure 1, Supplementary File). Among the two dependent variables, the target variable "Cured" was a binary variable that described whether the animal cured up to 12 days after metritis diagnosis. As for the 22 independent variables, we applied standard data preprocessing pipeline of feature scaling (FS), missing value imputation (MVI), and outlier detection (OD) in the specified order using the scikit-learn Python package and missingpy Python library (https://github.com/epsilon-machine/missingpy). We tested three variations of FS (standard, minmax, and robust), three variations of MVI: KNN (Troyanskaya et al., 2001), MICE (Buuren et al., 2010), and miss forest (Stekhoven et al., 2012), and two variations of OD: isolation forest (Liu et al., 2008), and LOF (Breunig et al., 2000).

**Feature selection.** To find the optimal subset of features for the classifier, we performed recursive feature elimination (RFE) on the preprocessed data using the Python library Mlxtend (https://github.com/rasbt/mlxtend). For each step of RFE, we removed a single feature associated with the best classifier performance if it was removed, until only a single feature (most informative) remained. For selecting the optimal subset, we chose the smallest feature subset that was within one standard error of the cross-validation performance instead of the feature subset with the best cross-validation performance (described below). This parsimonious feature selection helps reduce the number of features as much as possible while retaining a reasonable performance, therefore reducing the cost of data collection, reducing the training time, and allowing for generalizability.

**Model selection pipeline.** The overall model selection pipeline included the following steps: data preprocessing, feature selection, up-sampling using SMOTE (Valdés et al., 2018), and classifier training. For up-sampling, we resampled only the minority class (not-cured) to match the number of the majority class (cured). Therefore, the final number of samples used for training the machine learning algorithms was 794 (397 from each class). Up-sampling was only applied to the training process. We trained seven classifiers of varying complexity (decision tree (Mitchell, 1997), Gaussian naïve Bayes (Witten et al., 2011), multinomial naïve Bayes (Domingos and Pazzani, 1997), SVC (Hu et al., 2016), AdaBoost (Morra et al., 2010), random forest (Ho, 1995; Breiman, 2001), and artificial neural network (Zafeiris et al., 2018). Therefore, the model selection pipeline had 126 possible combinations (3 FS, 3 MVI, 2 OD, and seven classifiers). We then performed a grid search to find the best hyperparameter of the classifier for that given combination. For evaluation, we performed 5-fold cross-validation, which consists of partitioning the data into five equally or nearly equally sized segments. Subsequently, five iterations of training and validation are performed such that within each iteration, a different segment of the data is used as testing data, while the other four segments are used for training the data. The classification performance measured the classifier accuracy, Se, Sp, PPV, NPV, precision, and F-score. Accuracy was calculated as the ratio between the sum of the true positive (TP) and true negative (TN) results and the sum of TP, TN, false positives (FP), and false negatives (FN). Precision was calculated as the ratio between TP and the sum of TP and FP. The false detection rate was calculated as the ratio between FP and the sum of FP and TP. F1-score was calculated using the formula (2 x Se x precision) / (Se + precision). The performance of the classifier was optimized using the F1 score.

**Recommender system.** A recommender system is a personalized recommendation to handle information overload problems and improve description of an outcome of interest (Lu et al., 2015). All results presented in the manuscript were based on the recommender system. Using the best model among the 126 combinations from the model selection pipeline, we built a recommender system that recommended which are the cows that will cure or not from metritis. We recommended setting the threshold θ to the difference in cured and not-cured cows' prior probabilities.

**Experimental scenarios.** We trained the machine learning models on four different subsets of the features to analyze different end-user case scenarios regarding data availability on individual cow daily milk production (Table 1). In scenario one, we assumed that milk production data were not available, but all other features were used. Scenario two had all fixed-features and milk production slope up to 5, 7, and 9 DIM features. Scenario three had all fixed-features and milk differences from the day before to metritis diagnosis categorical (MilkVar) and continuous (Vmilk) features. Scenario four had all fixed and milk production features.

## Results

### Descriptive statistics

The descriptive statistics regarding the number of cows cured, parity, dystocia, RFM, VLS, mean BCS5, mean DIM D0, the rectal temperature at enrollment, mean of milk production slope at 5 DIM, and mean of milk difference from the day before to the metritis diagnosis for cows cured and non-cured are presented in Table 2. The proportion of cows with vulvovaginal laceration was greater (P < 0.01) in the non-cured than in the cured group. Mean DIM D0 and milk production slope at 5 DIM were greater (P < 0.01) in cured than in the non-cured cows. The milk difference from the day before to the metritis diagnosis was different (P < 0.01) between the groups being positive for cured cows and a negative for non-cured cows (Table 2). The rectal temperature at enrollment was lower (P = 0.02) in cured cows than the non-cured herdmates. The remained variables analyzed were not different between cured and non-cured cows (Table 2).

### Association of cow-level and environmental factors with metritis cure

The outcomes of the initial univariable logistic regression models evaluating the association of categorical variables with metritis cure is presented in Table 3. Week of enrollment (P < 0.03), treatment (P < 0.01), VLC (P <0.01), VLS (P < 0.01), slope of milk production up to 5 DIM categorical (P = 0.02), and categorical milk difference from day before to metritis diagnosis (P < 0.01), were positively associated with metritis cure. Season (P = 0.12), month of enrollment (P = 0.18), parity (P = 0.60), dystocia (P = 0.18), RFM (P = 0.46), BCSC (P = 0.85) and fever (P = 0.16), were not associated with metritis cure. For the continuous variable, days in milk at enrollment (P < 0.01), rectal temperature at enrollment (P = 0.02), slope of milk production up to 5 DIM (P = 0.01), and milk difference from day before to metritis diagnosis (P < 0.01) were positively associated with metritis cure, whereas lactation number (P = 0.31) and BCS5 (P = 0.60) were not associated with metritis cure (Table 4).

The outcomes from the final multivariable logistic regression model evaluating the association between cow-related factors and metritis cure are presented in Table 5. Treatment affected (P < 0.01) the odds of metritis cure with CEF having 1.99 higher odds of cure than cows in CON group. Cows without vulvovaginal laceration had 1.91 higher odds of cure than cows with vulvovaginal laceration ($P < 0.01$). There was a tendency for an interaction between treatment and vulvovaginal laceration ($P = 0.06$). Cows without vulva laceration treated with ceftiofur had 3.28 higher odds of cure than cows with vulva laceration. In contrast, cows with vulvovaginal laceration treated with ceftiofur had no differences in cure when compared to control cows. Cows that developed metritis after the first week post-partum had 2.09 higher ($P = 0.05$) odds of being cured than cows that developed metritis in the first week post-partum. For rectal temperature, each degree Celsius above 39.4 ºC leads to a tendency ($P = 0.08$) of 0.68 lower odds to be cured than cows with rectal temperature ≤ 39.4 ºC. Cows that had reduced milk production from the day before to the metritis diagnosis had 0.46 lower odds to be cured than cows with moderate milk production increase ($P < 0.01$).

The multivariable ROC curve revealed an area under the curve (AUC) for the model of 0.69 (Figure 1). The AUC in the multivariable ROC for rectal temperature at D0, DIM D0, milk difference, and milk production slope 5 DIM were 0.55, 0.62, 0.62, and 0.56, respectively. The Se, Sp, PPV, and NPV for the multivariable ROC were, 0.98, 0.08, 0.75, and 0.08, respectively. The accuracy, precision, F1 for the multivariable ROC were, 0.75, 0.75, and 0.85, respectively.

Optimal thresholds for rectal temperature, DIM D0, milk difference from the day before to the metritis diagnosis, and slope of milk production up to 5 DIM to predict cure were determined using univariate ROC curves (Figure 2). Among cows that cured, the milk difference threshold that maximized the area under the curve (AUC = 0.61) was > 0.09 kg (% cows below threshold = 60%), for DIM (AUC = 0.62) the threshold was > 5 days (% cows below threshold = 21.6%), and the temperature cut point for metritis cure (AUC = 0.56) was ≤ 39.2 °C (% cows below threshold = 42%).

### Predictions of metritis cure from the machine learning models

The models' accuracy for scenarios one, two, three, and four were 66%, 72%, 71%, and 70%, respectively (Table 6). The random forest classifier had the best performance across all four scenarios, while the best overall preprocessing pipeline was standard scaling for FS, KNN for MVI, and LOF for OD (Table 6). Scenario 2, which was trained using all features except for Vmilk and MilkVar, had the best performance based on the parsimonious point, which is the model that provides the simplest explanation that fits for best results when more than one option to choose is available. Scenario 2 had a F1-score of 0.81, an accuracy of 0.72, a PPV (precision) of 0.78, a NPV of 0.50, a sensitivity of 0.85 (recall), a specificity of 0.39, and false discovery rate (FDR) of 0.22.

## Discussion

Our results demonstrate that the cure for metritis is associated with cow-level and environmental factors and that the use of machine learning algorithms needs to be optimized to become a feasible approach to identify cows with greater probability of metritis cure. The accuracies of classic multivariable model was 75% that was higher than the best machine learning scenario (72%). However, considering the limited number of cows and variables used for the current study, more potential for improvement is expected with machine learning algorithms known to have an advantage handling large datasets and number of variables (Grzesiak, et al., 2010). Advancing predictive models for cure is a strategy that aligns with a worldwide effort to reduce the use of antibiotics in food animal systems, contributing to reducing the antimicrobial resistance problem, both in animals and humans (CDC, 2020).

In the current study, our goals were to investigate cow-level and environmental factors associated with metritis cure integrating classic multivariable statistical models and machine learning algorithms, which were reported as an effective strategy for data mining and development of predictive tools for fertility and insemination outcomes (Shahinfar et al., 2012; Hempstalk et al., 2015), metabolic diseases (Xu et al., 2019) and mastitis (Hyde et al., 2020).

An interesting finding of our study was that calving-related disorders already known as risk factors for metritis such as parity, dystocia, body condition score at five DIM, and RFM, were not included in the final multivariable logistic regression model because we did not detect an association with metritis cure up to 12 days after metritis diagnosis. It is unclear why these variables were not associated with cure by traditional logistic regression. Machado et al. (2020) found no association between parity and cure of metritis at 12 days after metritis diagnosis, consistent with our findings. Also, Lima et al. (2014) observed that parity was not associated with cure up to 12 days after enrollment. However, it was associated with cure within the first week after the initial diagnosis. Also, calving-related disorders such as dystocia, twin calves, and RFM were negatively associated with cure at six days but not 12 days after enrollment. Parity may be associated with an early cure, but the current study focused on the final assessment for the cure at 12 days post-metritis diagnosis. That was the time point where the cure rate between treated and non-treated cows differed previously for data herein used (de Oliveira et al., 2020).

The vulvovaginal laceration was an important variable for metritis cure in both sets of analyses. Cows without vulvovaginal laceration had higher odds of curing of metritis than cows with vulvovaginal laceration. The relevance of vulvovaginal laceration and metritis cure in classical statistical and machine learning models suggest that this variable might be a more accurate predictor for metritis cure than calving-related disorders, which is a combination of RFM, dystocia, stillbirth, and twins. Each of the four components of calving-related disorders has been associated individually with metritis risk (Hossein-Zadeh et al., 2011). Still, their association with metritis cure is less clear and, as suggested by the current study, is not consistently detected across different analytical methods. Both vulvovaginal laceration and calving-related disorders can increase tissue damage, and necrotic cells release damage-associated molecules or molecular patterns (DAMPs) that increase the inflammatory response and delay healing (Healy et al., 2014). Perhaps the release of DAMPs is more consistent in cows with vulvovaginal laceration and helps explain why this variable was ubiquitously present in our models and analytical methods used in the current study.

Results from a pilot study suggested that leaving metritis cases that occurred within the first 5 DIM did not lead to negative outcomes (Sannmann et al., 2013) and only cows with fever at the metritis diagnosis would benefit of the antibiotic treatment. However, our results from the classic multivariable statistical and ROC curve analysis, in contrast with this recommendation suggest that cows developing metritis in earlier lactation are less likely to cure than cows developing metritis after the first week post-partum. A possible reason for these results is that cows developing metritis earlier may have compromised immune response necessary to achieve uterine pathogen clearance, which can lead to increased uterine pathogen load and toxins production and lower efficacy of antibiotics treatment. Also, in the current study cows with a rectal temperature above 39.4 ºC tended to have lower odds of being cured than cows with a rectal temperature ≤ 39.4 ºC.

The current study results revealed that cows having reduced milk production from the day before to the metritis diagnosis are less likely to cure than cows with moderate milk production. Machine learning and logistic regression analyses indicated that milk production is an important variable for the predictability of a metritis cure. In the present study, the machine learning analysis was trained on four different subsets of variables to create different scenarios. When milk production was not included in the analysis, machine learning predicted metritis cure with an accuracy of 0.66. However, when the different milk production measurements were added to the analysis in the other three scenarios, the metritis cure predictability increased with an accuracy greater than 0.70. Traditional multivariable logistic regression analysis detected the slopes of milk production up to 5 DIM as the best variable to predict metritis cure, and the inclusion of only this variable generated a full model with the lowest AIC. Machine learning analysis indicated that the model including slopes of milk production for 5, 7, and 9 DIM, had the best accuracy to predict which cows cured of metritis, but the accuracy was still lower than the multivariate ROC analysis. The decrease in milk production with the onset of metritis can be attributed at least in part to a concomitant decrease in dry-matter intake associated with inflammation (Plata-Salamán et al., 1996; Pérez-Baez et al., 2019). Merenda et al. (2021) demonstrated that metritis profoundly affects inflammatory response, feeding, and rumination times compared with healthy cows. Immune activation markedly disrupts glucose homeostasis and is characterized by hypoglycemia and hyperlactemia (Filkins, 1978; McGuinness, 2005; Michaeli et al., 2012). The energy cost to keep the immune system activated under inflammation is very high (1,553 g for 720 minutes (Kvideira et al., 2017). Therefore, it is reasonable to surmise that cows experiencing metritis cure failure might exacerbate inflammatory response and activation of the immune system.

For most of the variables analyzed in the current study for an association or prediction of metritis cure, we could not see differences between cows treated and not treated for metritis. The only exception was a tendency for interaction between treatment and vulvovaginal laceration. The efficacy of ceftiofur to treat metritis is well established (Chenault et al., 2004; McLaughlin et al., 2012; de Oliveira et al., 2020). Cows without vulvovaginal laceration treated with ceftiofur had higher odds of curing of metritis than cows with vulvovaginal laceration, whereas a similar relationship was not found in the CON group. We speculate that animals with vulvovaginal laceration may have the severity of metritis increased, and the ceftiofur effectiveness to cure metritis might be diminished. Silva et al. (2021) reported in a recent study that cows with metritis that received an antibiotic treatment had lesser proportion of culling and similar mortality up to 300 DIM when compared with untreated. The data from Silva et al., (2021) suggest that increased culling rate may be related to the decreased milk production and reproductive performance experienced by non-treated metritic cows. Identifying cow-level and environmental factors leading to metritis cure might be a critical step to design a decision tree for selective treatment that goes beyond cure rate and positively impacts fertility and milk yield performance.

The machine learning also selected all variables included in the multivariable logistic regression recommended system as a metritis cure predictor. Furthermore, machine learning analysis pointed out calving-related disorders as an important feature for metritis cure prediction, indicating that these variables are useful and the development of a selective therapy for metritis using cow-related factors is a possibility. Using the AdaBoost algorithm, we were able to identify cow-level and environmental factors that predict metritis cure with an accuracy of up to 72%. Using the subset of variables selected by scenario two (all fixed-features and milk production slope up to 5,7, and 9 DIM) in the machine learning models, 72% of accuracy was achieved to determine whether the cow will cure or not from metritis. Ideally, an algorithm to be used to select cows to optimize cure without compromising animal welfare needs to have high sensitivity, high PPV, and moderate specificity. Our machine learning models had sensitivities ranging from 0.81-0.85 and PPVs ranging from 0.75-0.78, which suggest a good prediction towards judicious antimicrobial use. On the other hand, the specificities ranged from 0.26-0.39 suggesting that from animal welfare standpoint algorithms need to be improved. A major limitation of the present study is that the data used for the analysis is only from three herds, and that the present model was not validated using external data from other herds across the US and with differences in metritis prevalence. Validation of the results of the current study could be done by applying the algorithm in a further cohort field study in which cows would be selected to receive or not treatment based on the algorithm. Nonetheless, our work demonstrates that it may be possible to identify cows with greater probability of cure. Further studies would be necessary to improve the prediction ability of the model and it may be achieved by including other markers of inflammation and immunomodulation and other cow-related factors such as genomics, rumination, activity level, and dry matter intake.

## Conclusion

Overall, our multivariable logistic regression and ROC analysis indicates that cows developing metritis > 7 DIM, with increase in milk production, and with a rectal temperature ≤ 39.40 oC have increased likelihood of cure of metritis with an accuracy of 75% and sensitivity of 98%, which were both slightly higher than the values reported in our machine learning algorithms scenarios. Our findings from machine learning showed that besides these variables, consider calving-related disorders (dystocia, RFM, calf gender, twins, and stillbirth), season, the month of metritis event, and milk production slope up to 5, 7, and 9 DIM are helpful to predict whether the cow will cure or not from metritis. There is tremendous pressure on agriculture to improve the judicious use of antibiotics and metritis has been shown to be one of the major drivers of antimicrobial drug usage in lactating cows, underscoring the importance of predicting metritis cure. The unique capacity of machine learning algorithm handling large dataset and number of variables may contribute to advance the development of selective treatment for metritis, potentially reducing the dissemination of antimicrobial resistance, concurrent with lower disease cost and improved animal welfare.

Table 1-1. Comparison of the machine learning experiments with four different subsets of milk production-related features while keeping the other 18 independent fixed features. All different pairwise scenarios are statistically significant (P-value ≤ 0.0001)

| Scenarios | Features | | | | | Preprocessing | | | Best Classifier | F1-score |
|---|---|---|---|---|---|---|---|---|---|---|
| | Milk increase 5DIM | Milk increase 7DIM | Milk increase 9DIM | Vmilk | MilkVar | Feature Scaling (FS) | Missing Value Imputation (MVI) | Outlier Detection (OD) | | |
| One | X[1] | X | X | X | X | Standard | KNN | Isolation Forest | RandomForest | 78.4% |
| Two | O[2] | O | O | X | X | Minmax | Iterative | LOF | RandomForest | 81.2% |
| Three | X | X | X | O | O | Robust | Missforest | LOF | RandomForest | 81.1% |
| Four | O | O | O | O | O | Standard | KNN | LOF | RandomForest | 79.4% |

[1] X: Variable was not used in the analysis

[2] O: Variable was used in the analysis

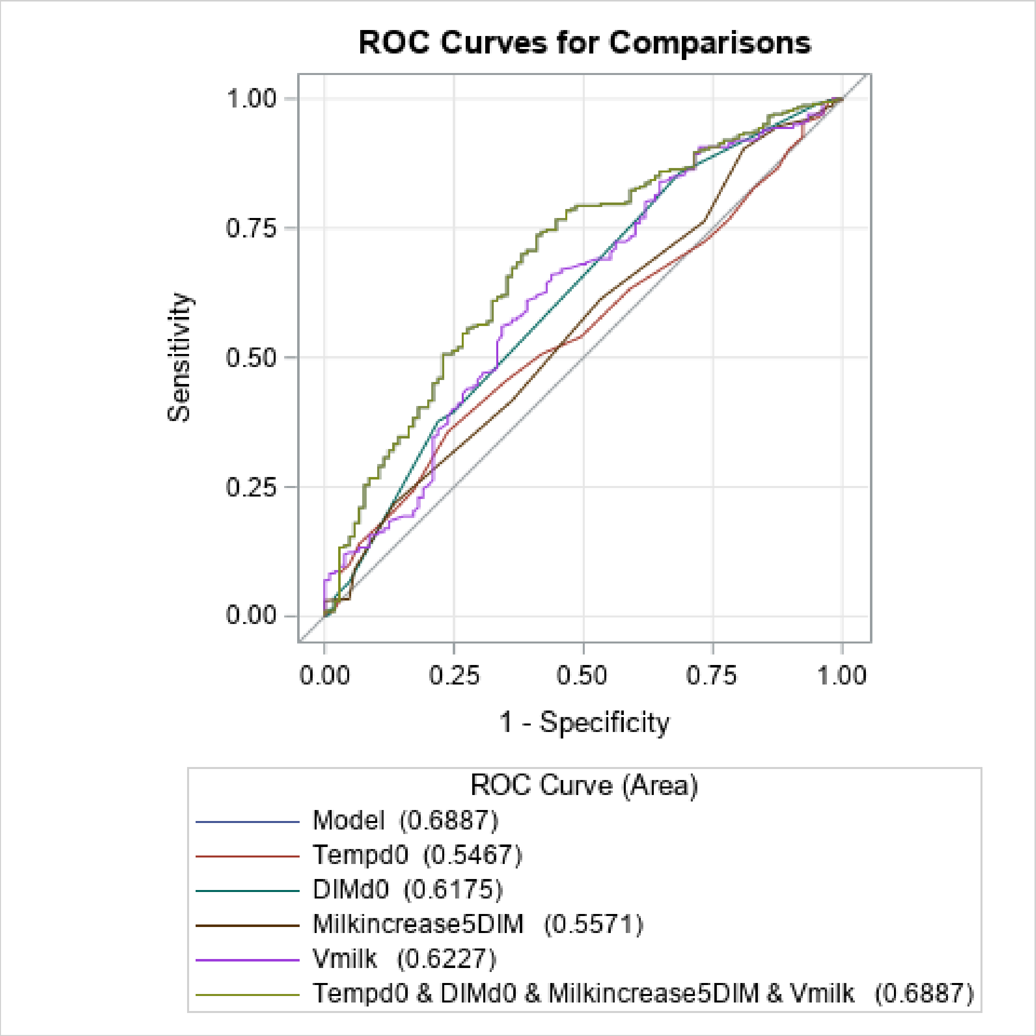


Figure 1-1. Comparison of receiver operating characteristic (ROC) curves with optimal threshold values for the whole model. Tempd0 = rectal temperature at metritis diagnosis; DIMd0 = DIM at metritis diagnosis; Milkincrease5DIM = slope of milk increase up to 5 DIM; Vmilk = milk production difference from day before to diagnosis.

Table 1-2. Descriptive statistics of cured and non-cured groups used in logistic regression and machine learning analysis.

| Item | Cured | | *P* |
|---|---|---|---|
| | YES | NO | |
| Number of animals enrolled (%) | 397 (72.2) | 153 (27.8) | |
| Number of primiparous animals enrolled (%) | 207 (72.4) | 79 (27.6) | 0.60 |
| Number of multiparous animals enrolled (%) | 190 (72.0) | 74 (28.0) | |
| Number of dystocia (%) | 112 (28.2) | 55 (35.9) | 0.18 |
| Number of animals with RFM (%) | 164 (41.3) | 65 (42.5) | 0.46 |
| Number of animals with vulvovaginal laceration (%) | 90 (22.7) | 56 (36.6) | <0.01 |
| Mean milk production slope 5 DIM[1] | 2.07 | 1.38 | <0.01 |
| Mean milk difference (Kg)[2] | 1.29 | -1.50 | <0.01 |
| Mean body condition score at 5 DIM | 3.43 | 3.42 | 0.88 |
| Mean DIM at metritis diagnosis | 7.6 | 6.9 | <0.01 |
| Mean rectal temperature at enrollment (°C) | 39.3 | 39.5 | 0.02 |

[1] Mean milk production slope 5 DIM: Mean of milk production slope up to 5 DIM

[2] Mean milk difference (Kg): Mean milk production from the day before to the metritis diagnosis continuous.

Figure 1-2. Optimal threshold values for rectal temperature at metritis diagnosis, DIM at diagnosis, milk difference from the day before diagnosis to the metritis diagnosis, and slope of milk increase up to 5 DIM. Outcome of 4 receiver operating characteristic curve analyses were performed to evaluate the optimum threshold of rectal temperature at metritis diagnosis (A), DIM at metritis diagnosis (B), milk increase (C), and milk slope associated with metritis cure (D). Se = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value. AUC = area under the curve

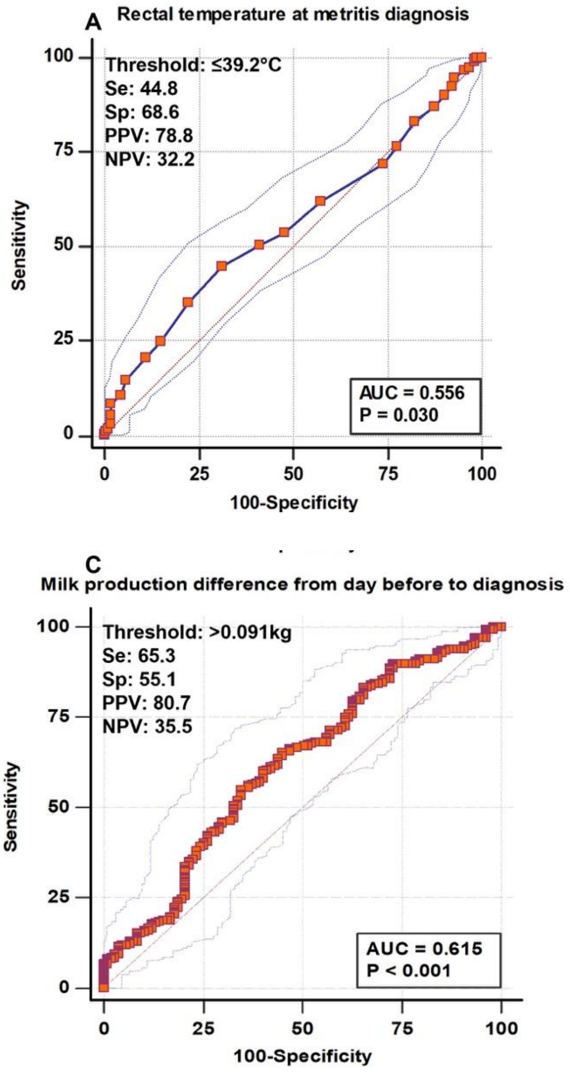


Figure 1-2. Continued

Table 1-3. Univariate logistic regression model evaluating the association of several categoric variables with metritis cure.

| Variable | Level | Odds ratio (95% CI) | *P* |
|---|---|---|---|
| BCSC | Low | 0.87 (0.48 – 1.59) | 0.85 |
| | Moderate | *Baseline* | |
| | High | 1.06 (0.66 – 1.70) | |
| Dystocia | Yes | *Baseline* | 0.18 |
| | No | 1.31 (0.88 – 1.94) | |
| Fever | Yes | *Baseline* | 0.16 |
| | No | 1.30 (0.89 – 1.89) | |
| Milk difference | Negative | 0.41 (0.25 – 0.70) | < 0.01 |
| | Moderate | *Baseline* | |
| | High | 0.84 (0.45 – 1.57) | |
| Milk production slope 5 DIM | Low | 0.78 (0.49 – 1.23) | 0.02 |
| | Moderate | *Baseline* | |
| | High | 1.88 (0.99 – 3.60) | |
| Parity | Primiparous | *Baseline* | 0.60 |
| | Multiparous | 1.11 (0.74 – 1.66) | |
| RFM | Yes | *Baseline* | 0.46 |
| | No | 1.15 (0.78 – 1.71) | |
| Season | Hot | *Baseline* | 0.12 |
| | Cool | 0.67 (0.41 – 1.12) | |
| Treatment | Control | *Baseline* | <0.01 |
| | Ceftiofur | 2.11 (1.44 – 3.11) | |
| VLC | Yes | *Baseline* | <0.01 |
| | No | 2.13 (1.41 – 3.22) | |
| VLS | Score 0 | 2.46 (1.51 – 4.03) | <0.01 |
| | Score 1 | 1.89 (1.18 – 3.01) | |
| | Score 2 | *Baseline* | |
| Week of enrollment | 1st week post-partum | 0.49 (0.32 – 0.74) | 0.03 |
| | 2nd week post-partum | *Baseline* | |

Body condition score at day five post-partum was categorized (BCSC) as Low ≤ 3.0, Moderate > 3.0 or < 3.75, or High ≥ 3.75; calving score (1 = unassisted, 2 = easy pull or slight problem, 3 = moderate pull; and 4 hard pull) was used to determine dystocia (No: calving score = 1 vs. Yes: calving score > 1); fever at the metritis diagnosis was categorized as Yes ≥ 39.5 °C vs. No ≤ 39.5 °C; milk slope up to 5 DIM and milk difference were categorized based on the percentile (P) of milk production increased obtained from the linear regression (low = P1 – P25; moderate = P25-P75, or high > P75), milk production from the day before to the metritis diagnosis categoric (MilkVar); parity was categorized as primiparous vs. multiparous; retained of fetal membranes (RFM) were categorized as Yes and No; vulvovaginal laceration (VLS) was categorized as 0 = no laceration; 1 = laceration < 2 cm at dorsal commissure or internal vaginal wall; 2 = vulvovaginal laceration > 2 cm; categorical VLS (VLC) was further divided into Yes ≥ 2cm or No < 2 cm.

Table 1-4. Univariate logistic regression model evaluating the association of several continuous variables with metritis cure.

| Variable | Level | Odds ratio (95% CI) | *P* |
|---|---|---|---|
| Days in milk at enrollment | 7 DIM | *Baseline* | <0.01 |
| | 8 DIM | 1.17 (1.06 – 1.28) | |
| Milk production slope 5 DIM | 1.89 | *Baseline* | 0.01 |
| | 2.89 | 1.11 (1.02 – 1.21) | |
| Milk difference | 0.57 kg | *Baseline* | <0.01 |
| | 1.57 kg | 1.07 (1.03 – 1.11) | |
| Number of lactations | 2 lactations | *Baseline* | 0.31 |
| | 3 lactations | 1.09 (0.92 – 1.27) | |
| Rectal temperature | 39.4 °C | *Baseline* | 0.02 |
| | 40.4 °C | 0.64 (0.44 – 0.93) | |

Table 1-5. Outcomes from the final multivariable logistic regression model evaluating the association between cow related factors and metritis cure.

| Variable | Level | Odds ratio (95% CI) | *P* |
|---|---|---|---|
| Milk difference | Reduced (n = 161) | 0.46 (0.27 – 0.79) | < 0.01 |
| | Moderate (n = 151) | *Baseline* | |
| | High (n = 103) | 0.92 (0.48 – 1.78) | |
| Temperature | 39.40 (n = 287) | *Baseline* | 0.08 |
| | 40.40 (n = 263) | 0.68 (0.43 – 1.06) | |
| Treatment | CON (n = 275) | *Baseline* | < 0.01 |
| | CEF (n = 275) | 1.99 (1.21 – 3.26) | |
| VLC | Yes (n = 403) | *Baseline* | < 0.01 |
| | No (n = 147) | 1.91 (1.17 – 3.14) | |
| Week postpartum | 1 (n = 343) | 0.49 (0.29 – 0.81) | < 0.01 |
| | 2 (n = 207) | *Baseline* | |

Vulvovaginal laceration (VLS) was categorized as 0 = no laceration; 1 = laceration < 2 cm at dorsal commissure or internal vaginal wall; 2 = vulvovaginal laceration > 2 cm; categorical VLS (VLC) was divided into Yes ≥ 2cm laceration or No < 2 cm laceration.

Table 1-6. Performance of machine learning algorithms for scenarios 1, 2, 3 and 4. Minmax feature scaling, iterative missing value imputation, LOF outlier detection, and random forest classifier were used to produce these results. All scenarios model included the final number of samples used for training the machine algorithms that was up-sampled to correct inbalance (n = 794, cured = 397 and non-cured = 397).

| | **Scenarios [3]** | | | |
|---|---|---|---|---|
| | Scenario 1 | Scenario 2 | Scenario 3 | Scenario 4 |
| Sensitivity | 0.81 ± 0.03 | 0.85 ± 0.03 | 0.85 ± 0.04 | 0.81 ± 0.03 |
| Specificity | 0.26 ± 0.07 | 0.39 ± 0.07 | 0.35 ± 0.3 | 0.39 ± 0.07 |
| PPV[1] | 0.75 ± 0.02 | 0.78 ± 0.02 | 0.77 ± 0.01 | 0.78 ± 0.02 |
| NPV[2] | 0.33 ± 0.06 | 0.50 ± 0.04 | 0.49 ± 0.09 | 0.45 ± 0.06 |
| Accuracy | 0.66 ± 0.03 | 0.72 ± 0.02 | 0.71 ± 0.03 | 0.70 ± 0.03 |
| F1-score | 0.78 ± 0.02 | 0.81 ± 0.02 | 0.81 ± 0.02 | 0.79 ± 0.02 |
| False detection rate | 0.25 ± 0.02 | 0.22 ± 0.02 | 0.23 ± 0.01 | 0.22 ± 0.02 |

[1] PPV: Positive predictive value

[2] NPV: Negative predictive value

[3] Scenarios: Scenario 1: none of the milk production features were used; Scenario 2: Milkincrease5DIM, Milkincrease7DIM, and Milkincrease9DIM features were used along with 18 other features; Scenario 3: Vmilk and MilkVar features were used along with 18 other features; Scenario 4: all 5 milk production features were used along with 18 other features.

# CHAPTER 2
# PREDICTING SPONTANEOUS METRITIS CURE IN LACTATING DAIRY COWS: DESIGNING A DECISION TREE.

## Abstract

The study objectives were to identify cow-level and environmental factors associated with spontaneous metritis cure (SMC) and evaluate traditional statistical methods and machine learning algorithms (ML) ability to predict SMC. A subset of 438 primiparous and multiparous lactating Holstein cows from two studies in Florida, Texas, and California that remained untreated after metritis diagnosis were used for the study. Cows were diagnosed with metritis at 5, 7, and 9 days in milk using the Metricheck® device. Cows with fetid, watery, reddish-brown uterine discharge, with or without necrotic tissue, were classified as having metritis. The SMC was defined as an absence of fetid, watery, reddish-brown uterine discharge up to 14 days after enrollment. Cow and environmental level data were offered to the univariate logistic regression models to predict SMC, and variables with $P < 0.10$ in univariate models were included in a multivariable logistic regression model. A multivariable ROC curve analysis was performed for significant variables in the multivariable logistic regression models to evaluate diagnostic test and the SMC predictive model sensitivity and specificity. Cows developing metritis after the first week postpartum had 1.24 times higher odds of SMC than cows having metritis within the first week postpartum ($P < 0.01$). For each one degree Celsius increase above 39.3°C, a 0.63 lower odds of SMC ($P = 0.02$) was observed. For each kilogram of milk production increase above 0.91 kg starting the day before metritis diagnosis a 1.05 greater odds of SMC ($P < 0.01$) was observed. The multivariable ROC curve revealed an area under the curve (AUC), accuracy, precision, and F1 score of 0.68, 0.66, 0.65, and 0.78, respectively. For the ML, the Lasso decision tree was the best classifier for SMC and the main variables relevant to SMC predictability were days in milk of metritis diagnosis and rectal temperature (accuracy of 63%). The results from multivariable logistic models suggest that cows developing metritis after the first week postpartum, cows with increasing milk production the day before to metritis diagnosis, and cows with a rectal temperature ≤ 39.3°C at the time of the metritis diagnosis have an increased odds of SMC, ROC multivariable analysis accuracy was of 63%.The ML showed the same accuracy (63%) even if using only milk difference from the day before to the metritis diagnosis and rectal temperature on metritis diagnosis day for SMC predictability or if adding milk difference from the day before to the metritis diagnosis to the SMC. Overall, logistic regression models and machine learning showed a promissing potential in predicting SMC, and using only a few variables in the analysis. SMC prediction can contribute to advance the development of selective antimicrobial drug treatments for metritis, potentially reducing the dissemination of antimicrobial resistance, concurrent with lower disease cost and improved animal welfare.

**Key Words:** Machine learning, spontaneous metritis cure, dairy cow

## Introduction

Metritis is an acute uterine disease during the postpartum characterized by the presence of fetid, watery, reddish-brownish vaginal discharge (Chenault et al., 2004). In the United States, the incidence of metritis ranges from 20 to 35% (Pinedo et al., 2020), and cows having calving-related disorders such as dystocia, twin calves, stillbirth, vulva laceration, and retained fetal membranes (RFM) can have increased incidence ranging from 30% to 45% (Vieira-Neto et al., 2016). Moreover, metritis is commonly associated with decreased pregnancy per artificial insemination, increased culling rates, and reduced animal welfare (Silva et al., 2021).

The cost of metritis varies according to the strategy adopted for treatment; even using different protocols is costly and drives a significant economic impact on dairy farms. In the United States, the cost of metritis varied from $156 to $ 948 depending on the scenario assessed (Silva et al., 2021; Pérez-Báez et al., 2021; Lima et al., 2019). In studies comparing the cure-risk of cows treated with ceftiofur and non-treated metritic cows, more than half (55-62%) of metritis cases in the non-treated group of cows undergo spontaneous cure (Chenault et al., 2004; McLaughlin et al., 2012; de Oliveira et al., 2020). Additionally, ceftiofur treatment failure occurs in 22-25%. Considering that the additional clinical cure when receiving antimicrobial treatment is only 20%, it remains unclear if all cows curing are benefiting from the therapy or if the benefits are restricted to a portion of the cases. Thus, a predictive model that identifies cows at greater risk of metritis spontaneous cure, which do not require antibiotic treatment, is needed to guide antimicrobial decisions, and optimize the use of antibiotics on dairy farms. The feasibility of using metritis self-cure prediction to guide antimicrobial treatment decisions will depend on the economics and the drawbacks of antimicrobial resistance (AMR) development associated with metritis therapy. Some researchers (de Oliveira et al., 2020; Merenda et al., 2021) have demonstrated that cows diagnosed and treated for metritis have lower pregnancy rates and milk yield compared with healthy cows. Additionally, Silva et al. (2021) reported an estimated economic return of $207 per cow when using ceftiofur as a treatment alternative for metritis in dairy cows. However, it is unknown if metritis cases that undergo spontaneous cure have a decreased impact on these economically important outcomes. Additionally, data on the development of AMR associated with metritis treatment with ceftiofur is scarce.

Epidemiological data indicate that the definition and strategy for the diagnosis of metritis are not standardized across dairy farms (Espadamala et al., 2018). Additionally, because of the high prevalence of this disease, within the dairy industry, the treatment of metritis is one of the major drivers of antimicrobial drug usage in lactating cows (Kaniyamattam et al., 2020). Judicious use of antibiotics is critical for agriculture sustainability (Manyi-Loh et al., 2018). Antimicrobial resistance has become a major public and animal health concern. Also, because ceftiofur belongs to a class of antimicrobial drugs considered the highest priority for public health by the World Health Organization (WHO, 2020), there is a critical need for reducing its usage in dairy cows. Minimizing the use of antimicrobials in food-producing animals is expected to reduce the number of residue violations in milk and meat and reduce the risk of increased antimicrobial resistance of certain classes of drugs, such as the third generation of cephalosporin that can share common AMR with similar drugs used in human medicine (Fair et al., 2014).

Machine learning algorithms are acknowledged as powerful tools for predictive classification in a large dataset such as in agriculture-related studies (Grzesiak et al., 2010). They have been used to predict important events related to dairy cows, such as parturition, dystocia, and disease events (Borcher et al., 2017; Kovacs et al., 2017, Shahinfar et al., 2012). Recently, Oliveira et al., (2021) demonstrated that days in milk and rectal temperature at metritis diagnosis, an increase in milk production up to five days in milk, and vulvovaginal laceration were associated with an increased odds of metritis cure, but the authors reported that the study was unable to replicate the potential benefit of ML to optimize a predictive model of metritis cure in response to antimicrobial treatment. Furthermore, Machado et al., (2020) reported that the circulating concentration of haptoglobin and days in milk at metritis diagnosis is associated with the risk of a spontaneous cure for metritis. Additionally, vulvar laceration and dystocia could increase the chance of ceftiofur treatment failure. Although these results support the hypothesis that it may be possible to predict the risk of cure from metritis, there is a knowledge gap on self-cure in metritis and information that could help to reduce the unnecessary antibiotic treatment of cows with metritis when it can be avoided. Predictive models that identify cows at greater risk of spontaneous cure from metritis need to be explored.

The first objective of this study was to identify cow-level and environmental factors associated with the likelihood of SMC. The second objective was to evaluate the ability of traditional statistical models and ML to predict SMC. Our premise is that cow and environmental-related factors can predict metritis spontaneous cure with traditional statistical models and ML.

## Materials And Methods

### Data and animals

The cows used in this study are a subset of cows used in a larger experiment that compared the efficacy of different therapies and metritis cure (de Oliveira et al., 2020), and a subset of cows from a trial to identify cow level and environmental factors measured at the time of metritis diagnosis that is associated SMC. Briefly, the study from Oliveira et al., (2020), was conducted in three dairies located in North Central Florida from May 2016 to June 2017, and the trials to evaluate factors associated with the SMC were conducted in one dairy located in central valley California (4,300 lactating Holstein cows) and one dairy in northwest Texas (2,900 lactating Holstein cows) from August to October 2020 and January 2020 to May 2021, respectively. All herds milked only Holstein cows. The rolling herd average milk yield ranged from 10,150 to 12,000 kg. Postpartum pens had sand-bedded stalls and were equipped with sprinklers over the feeding areas that were activated when the environmental temperature rose above > 21 °C. The postpartum diet was formulated to meet or exceed the dietary nutrient requirements for a lactating cow weighing 680 kg and producing 45 kg of 3.5% fat-corrected milk and 3.0% protein (NRC, 2001), and it was delivered as a TMR twice daily.

Cows were diagnosed for metritis at 5, 7, and 9 days in milk (DIM) using the Metricheck® device (Simcro, Hamilton, NZ). Cows with fetid, watery, and reddish-brownish discharge, with or without pieces of necrotic tissue present, were classified as having metritis (Sheldon et al., 2006). Because we were interested in evaluating metritis spontaneous cure, only cows that did not received any antibiotic treatment control group (CON) were included in the present study (n = 438). At 5 DIM, all cows were scored for body condition (BCS; 1 = thin, 5 = obese; Ferguson et al., 1994) and vulvovaginal laceration (VLS; 0 = no laceration; 1 = laceration < 2 cm at dorsal commissure or internal vaginal wall; 2 = laceration > 2 cm) (Vieira-Neto et al., 2016). The vulvovaginal laceration was categorized (VLC) into No (< 2 cm) or Yes (> 2 cm). Rectal temperature was measured at enrollment, and cows with a rectal temperature ≥ 39.5 ºC were considered febrile. Additionally, the occurrence of dystocia, twins, RFM, and stillbirth were recorded. The criterion to determine SMC was an absence of the characteristic fetid, watery, reddish-brownish discharge at twelve days after enrollment. Moreover, a cow was considered as non-cured if she was sold or died before day twelve after metritis diagnosis.

Cows that presented severe dehydration, anorexia, weakness, severe depression, systemic shock, or any other clinical signs attributable to metritis (other than characteristic vaginal discharge and fever) received antibiotic therapy according to the farm protocol and were considered as non-cured. Milk yield, DIM to pregnancy and culling in the study were also recorded. Additional diseases were not accounted in the analysis.

### Reproductive Management and Pregnancy

For the herds in the different states, the voluntary waiting ranged from 48 to 77 DIM. Cows were artificially inseminated when detected in estrus by visual observation or using a heat detection device (Kamar ®, Kamar Inc., Steamboat Springs, CO). In two herds in Florida, cows that had not been inseminated by heat detection were submitted to the OvSynch protocol and were fixed-time AI. The Other herd in Fl and the herds in TX and CA, cows that had not been inseminated by heat detection were submitted to the Double-OvSynch protocol and were fixed-time AI.

The diagnoses of pregnancy were performed by transrectal ultrasonography (Easi-Scan linear bovine ultrasound machine, IMV Imaging, North America, Inc., Rochester, MN) at 33 ± 3 d after service. Nonpregnant cows were resynchronized using the OvSynch protocol. All the breeding and pregnancy diagnoses were recorded on the on-farm management software by the herd personnel. Weekly backups were saved and used to retrieve reproductive performance data. Pregnancy per breeding to first service and time to pregnancy up to 300 DIM were calculated for all cows enrolled in the experiment.

### Statistical Analysis

From the data collected in the studies described above, we defined, categorized, and used the following variables for every cow enrolled in the study: DIM D0 (days in milk at metritis diagnosis), categorical week at metritis diagnosis (metritis diagnosed at the first 7 days post-partum vs. diagnosed after seven days post-partum), season of the metritis diagnosis (cool: September to May vs. hot: June to August), month of metritis diagnosis (January – December), lactation number, parity (primiparous vs. multiparous), calving score (1 = unassisted, 2 = easy pull or slight problem, 3 = moderate pull; and 4 hard pull), dystocia (No: calving score = 1 vs. Yes: calving score > 1), RFM (yes vs. no), body condition score at day five post-partum (BCS5), categorical BCS5 (BCS5C, Low < 3.0, Moderate ≥ 3.0 or ≤ 3.5, or High > 3.5), VLS (vulvovaginal laceration score; 0, 1 or 2), categorical VLS (VLC, > 2 cm vs. < 2 cm), rectal temperature at the metritis diagnosis, fever at the metritis diagnosis (Yes ≥ 39.5 °C vs. No ≤ 39.5 °C), milk production from the day before to the metritis diagnosis continuous (Vmilk), milk production from the day before to the metritis diagnosis categoric (Milk P), and the slope of milk production up to 5, 7 and 9 DIM. The slopes of milk production up to 5, 7, and 9 DIM were calculated using linear regression models (PROC REG, SAS 9.4; SAS Institute Inc., Cary, NC, USA). The model included daily milk production (kg) as a dependent variable and days in milk as an independent variable. Milk production from the day before to the metritis diagnosis categoric and slope of milk increase was categorized based on the percentile (P) of milk production obtained from the linear regression (low = P1 – P25; moderate = P25-P75, or high > P75).

Descriptive statistical analysis regarding continuous data, such as DIM D0, the slope of milk production, milk difference, the rectal temperature at enrollment, and BCS at metritis diagnosis, were compared nonparametrically using the PROC NPAR1WAY of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA), and P-values from the Kruskal-Wallis's test are reported. Mean values for normally distributed continuous variables were generated using ANOVA to compare non-cured and cured groups. For the categorical variables (dystocia, parity, RFM, VLC), GLIMMIX procedure of SAS was used to compare cows that spontaneously cured or failed to cure from metritis and PROC FREQ of SAS was used to report proportions.

A three-step approach was used to select the variables to be included in the final multivariate models. First, univariable analyses for each independent variable were performed to assess their association with the odds of spontaneous metritis cure with the farm as a random effect (Oliveira et al., 2020), and variables were ranked according to their P-value. Second, multivariable analyses were performed using PROC MIXED and GLIMMIX with maximum likelihood and Laplace parameter estimation methods, respectively. All variables were offered to the model according to their P-value from the univariate analysis (smallest to largest), and the model Akaike Information Criteria (AIC) was used to select the final model variables (stepwise elimination approach). The model with the lowest AIC was used. Finally, PROC MIXED and GLIMMIX were used with restricted maximum likelihood (REML) and residual subject-specific pseudo likelihood (RSPL) default specifications for parameter estimation. The model for the multivariate analysis included cure as a dependent variable and the effects of days in milk at the metritis diagnosis, dystocia, rectal temperature at metritis diagnosis and milk production from the day before to the metritis diagnosis continuous.

The multivariable analysis results are reported as the ratio of the odds of metritis cure for each risk factor. The unit of analysis was the cow. Statistical significance was declared for all models described above if $P \leq 0.05$, and a tendency was considered if $0.05 < P < 0.10$. For variables that were significant in the multivariate logistic regression model, a multivariable ROC curves analysis was performed using the logistic procedure of SAS (PROC LOGISTIC, SAS 9.4; SAS Institute Inc., Cary, NC, USA). The results of ROC were reported as sensitivity (Se), specificity (Sp), positive predicted value (PPV), and negative predicted value (NPV) and were considered as follows: less than 0.2 as low, 0.2 to 0.4 as fair, 0.4 to 0.6 as moderate, 0.6 to 0.8 as substantial, and above 0.8 as high (Dohoo et al., 2009).

### Analysis for ML

The ML analysis was used to predict cows that would spontaneously cure or not from metritis. The data set contained 438 observations and 22 independent variables. Variable selection was made based on the β-coefficient for each variable using linear regression. To find the optimal subset of variables for the classifier, we performed recursive feature elimination on the preprocessed data using the Julia package MLJ.jl (Blaom et al., 2020). Six classifiers of varying complexity were trained [EvoTree, Tuned Decision Tree, Lasso, Lasso Decision Tree, Ridge, and K-nearest neighbors]. λ was chosen through 5-fold cross-validation. To compare the models, we used receiver operating characteristics (ROC) curves to measure and compare the discriminative performances of our models. The area under the ROC curves indicates how well a prediction model discriminates between animals that cure or not. The values vary from 0.5 (random guess) to 1.0 (perfect accuracy). And finally, a decision tree was built to create the list of variables that will perform better in answering the binary question of self-cured or not.

## Results

### Descriptive statistics

The mean DIM at metritis diagnosis was greater ($P < 0.01$) in spontaneously cured than in the non-cured cows. The milk difference from the day before metritis diagnosis was positive for cured cows and negative for non-cured cows ($P < 0.01$; Table 1). The rectal temperature at enrollment was lower ($P < 0.01$) in cured cows than in the non-cured herdmates. Nevertheless, parity ($P = 0.50$), dystocia ($P = 0.06$), RFM ($P = 0.79$), VLS ($P = 0.11$), mean milk production slope up to 5 DIM ($P = 0.48$), and mean body condition score at 5 DIM ($P = 0.59$) were not different between spontaneously cured and non-cured cows (Table 1).

### Association of cow-level and environmental factors with metritis spontaneous cure

From the initial univariable logistic regression models evaluating the association of variables with metritis spontaneous cure, categoric variables such as week of enrollment ($P < 0.01$), calving score ($P = 0.03$), dystocia ($P = 0.04$), fever ($P = 0.01$), categoric milk difference from day before to the metritis diagnosis day ($P = 0.01$), and categoric slope of milk production up 7 DIM ($P = 0.04$) were associated with metritis spontaneous cure. Parity ($P = 0.91$), RFM ($P = 0.59$), VLC ($P = 0.13$), BCSC ($P = 0.95$), season ($P = 0.19$), month of enrollment ($P = 0.18$), and categoric slope of milk production up to 5 ($P = 0.49$) and 9 ($P = 0.95$) DIM were not associated with metritis spontaneous cure. For the continuous variable, days in milk at enrollment ($P < 0.01$), rectal temperature at enrollment ($P < 0.01$), and milk difference from the day before to metritis diagnosis ($P = 0.01$) were positively associated with metritis spontaneous cure, whereas lactation number ($P = 0.48$), BCS5 ($P = 0.95$) and slope of milk production up to 5 ($P = 0.88$), 7 ($P = 0.59$) and 9 ($P = 0.56$) DIM were not associated with metritis spontaneous cure.

The outcomes from the final multivariable logistic regression model evaluating the association between cow-related factors and metritis spontaneous cure are presented in Table 2. For days in milk of metritis diagnosis, each day above 7 DIM leads to 1.24 higher ($P < 0.01$) odds of metritis spontaneous cure. For rectal temperature, each degree Celsius above 39.3 ºC was associated with 0.63 ($P = 0.02$) lower odds of spontaneous metritis cure. Each kilogram of milk production increase above 0.91 Kg from the day before to the day of metritis diagnosis was associated with 1.05 greater ($P < 0.01$) odds of spontaneous metritis cure. Dystocia was not significant on spontaneous metritis cure in the final multivariable model ($P = 0.20$).

The multivariable ROC curve included the following variables: rectal temperature at D0 (AUC: 0.57), DIM D0 (AUC: 0.63), and milk difference from the day before to the day of metritis diagnosis (AUC: 0.59). The multivariable ROC curve revealed an area under the curve (AUC) for the model of 0.68 (Figure 1). The Se, Sp, PPV and NPV for the multivariable ROC were, 0.98, 0.11, 0.65, and 0.11, respectively. The accuracy, precision, F1 for the multivariable ROC were 0.66, 0.65, and 0.78, respectively.

### Predictions of metritis cure from machine learning analysis

The lasso decision three (AUC = 0.63) classifier had the best performance compared to lasso (AUC = 0.61), evoTree (AUC = 0.62), ridge (AUC = 0.62), tuned decision tree (AUC = 0.60) and Knn (AUC = 0.55) (Figure 2). Lasso decision tree had a F1-score of 0.71 and an accuracy of 0.65. Furthermore, lasso decision three led to a minimal number of features that better explained spontaneous metritis cure and the relevant features were the rectal temperature at the metritis diagnosis day and days in milk of metritis diagnosis (Figure 2). Additionally, in a scenario using the decision tree on the variables chosen by lasso (the rectal temperature at the metritis diagnosis day and days in milk of metritis diagnosis) and a fixed threshold of 20% false positives. The number of treatments avoided in cows that did not need treatment was 42,6%, and the number of animals not receiving treatment that should have been treated was 15.2%. The percentage of the population that would have self-cured was 62.1%, and the overall treatment reduction was 32.2% (Figure 3).

## Discussion

Our results demonstrate that cow-level and environmental factors such as days in milk of the metritis event, milk production, and rectal temperature at the metritis diagnosis day are associated with spontaneous metritis cure. Considering the number of variables and accuracy of both predictive models, multivariable logistic regression and machine learning analysis demonstrated potential benefits to select cows with a greater likelihood of SMC. A tool that can predict SMC with high accuracy can help farmers make a guided decision regarding antibiotic treatment, predict the severity of metritis, identify potential animals that will not respond to the antibiotic and consequently be able to intervene and cull animals during the early stage of lactation.

A study from de Oliveira et al., 2021 reported that days in milk and rectal temperature at metritis diagnosis, an increase in milk production up to five days in milk, and vulvovaginal laceration were associated with the odds of metritis cure. The ML showed that besides these variables, calving-related disorders (dystocia, RFM, calf gender, twins, and stillbirth), season, the month of metritis event, and milk production slope up to 5, 7, and 9 DIM were helpful in predicting whether the cow that will cure or not from metritis. However, even though both methods have presented substantial accuracy and were pointed out as promising for metritis cure prediction, the authors could not replicate the potential benefit of ML to predict spontaneous metritis cure due to the subset of animals receiving antimicrobial treatment that were used for the analysis. Therefore, identifying cow-level and environmental factors leading to spontaneous metritis cure might be a critical step in designing a decision tree for selective treatment. In the current study, our goals were to investigate cow-level and environmental factors associated with SMC using classic multivariable statistical models and ML analysis, which were reported as an effective strategy for data mining and development of predictive tools for fertility and insemination outcomes (Shahinfar et al., 2012; Hempstalk et al., 2015), metabolic diseases (Xu et al., 2019) and mastitis (Hyde et al., 2020).

An interesting finding of our study was that dystocia was included in the final multivariable logistic regression model, but other calving-related disorders also known as risk factors for metritis such as vulvar laceration and RFM were not associated with SMC. Additionally, parity and body condition score at five DIM were not included because an association with SMC up to 14 days after diagnosis was not observed. Those variables are inextricably linked to the risk of metritis, but the structure of the dataset is highly dependent on the specific population and the occurrence of each variable, which can explain one variable being relevant and others not for a given population. Parity may be associated with an early MSC, but the current study focused on the final assessment for the cure at 14 days post-metritis diagnosis. Machado et al. (2020) found no association between parity and cure of metritis at 12 days after metritis diagnosis, consistent with our findings. Oliveira et al. (2021) in a study assessing metritis cure and considering animals receiving ceftiofur treatment, reported that some of the calving-related disorders were not associated with metritis cure up to 12 days after enrolment as well, but cows having vulvar laceration would take benefit of ceftiofur treatment and it would be an important variable to consider for metritis cure prediction in the scenario for the animals that will receive antibiotic treatment for metritis. Additionally, Lima et al. (2014) observed that parity was not associated with MSC up to 12 days after enrollment. However, it was associated with MSC within the first week after the initial diagnosis. Additionally, Lima et al. (2014), reported that calving-related disorders such as twin calves, and RFM were negatively associated with cure at six days but not 12 days after enrollment.

Multivariable statistical and ROC curve analysis, revealed that, overall, days in milk of metritis event, fever and milk production are associated with MSC. Oliveira et al. 2021, reported similar variables as relevant to the prediction of metritis cure for both multivariable logistic regression model and machine learning algoritm, using groups of animals curing from metritis and receiving antibiotic treatment or remaining untreated. In addition to the variable milk difference from the day before the metritis diagnosis, data related to milk production (milk slope up to 5, 7 and 9 DIM) increased the predictability of metritis cure. Studies focusing on metritis cure for animals receiving antibiotic treatment and remaining untreated have been reported same results. (de Oliveira et. al., 2021; Machado et al., 2020). The main point regarding the similarity of variables on either general or self-cure might be related to the disease severity, in which animals having severe metritis cases have lower likelihood of cure either receiving or not antibiotic treatment, whereas animals having less severe cases have a high likelihood of MSC.

The combination of the variable’s rectal temperature at D0, DIM D0, and milk difference from the day before to the day of metritis diagnosis that increased predictability of SMC, are biologically associated to immune response, tolerance, and pathogen bacteria proliferation. Cows developing metritis during the post-partum may have compromised immune response necessary to achieve uterine pathogen clearance, which can lead to increased uterine pathogen load and toxins production and increased disease severity. Fever has been positively correlated to the abundance of main pathogenic metritis bacteria such as Bacteroidetes and Fusobacteria (Jeon et. al. 2015, 2016b). Additionally, fever can be associated with increased haptoglobin concentrations which are associated to inflammatory response (Galvao et al., 2010; Nightingale et al., 2015). A more robust inflammatory response may lead to more tissue damage because of the generation and release of reactive oxygen species and the release of proteolytic enzymes, as reported by Lacy (2006), which may delay healing and clinical signs resolution. Also, the reduction in milk production can be attributed at least in part to a concomitant decrease in dry-matter intake associated with inflammation (Plata-Salamán et al., 1996; Pérez-Baez et al., 2019), and immune activation markedly disrupts glucose homeostasis and is characterized by hypoglycemia and hyperlactemia (Filkins, 1978; McGuinness, 2005; Michaeli et al., 2012). The energy cost to keep the immune system activated under inflammation is very high (1,553 g for 720 minutes (Kvideira et al., 2017). Therefore, it is reasonable to surmise that cows experiencing spontaneous metritis cure failure might exacerbate inflammatory response and activation of the immune system.

The multivariable ROC test diagnosis had high sensitivity, low specificity, low NPV and high PPV. Prevalence impact on PPV and NPV. As the prevalence increases, the PPV also increases but the NPV decreases (Kelly et al., 2010). In the present study, one explanation for the higher PPV when compared to NPV is possibly due to the high prevalence (65%) of SMC, our main outcome of interest.

Machine learning analysis may be an important tool to be used for implementing a selective treatment approach in dairy farms to predict SMC in post-partum cows, using the Lasso decision tree classifiers we were able to predict SMC with an accuracy of 71%. Different from the ROC multivariable logistic regression analysis (accuracy of 63 %), a smaller number of variables were used for the algorithm model and the decision tree on SMC prediction. Only considering rectal temperature and DIMD0, the final algorithm performed better than if milk production data was included. It highlights the flexibility of machine learning algorithms to make assumptions for parametric and non-parametric models through the understanding the structure of data and fit of the models, which in this case resulted in a model that required fewer variables to accurately predict SMC, which facilitates the implementation of such an approach in field conditions (Grzesiak et al., 2010). Another alternative to improve prediction of SMC could be using deep neural networks. Artificial neural networks and deep learning relies on numerous layers of neural network that decide classification and prediction. Different studies have reported that artificial neural networks provide higher accuracy than other machine learning algorithms (Sahoo et al., 2020; Doupe et al., 2019).

Using the lasso decision tree and having a scenario with a fixed threshold of 20% false positives for SMC, we were able to demonstrate a substantial reduction in antibiotic use for metritis treatment. The number of treatments avoided in cows that did not need treatment was 42.6% and the number of animals not receiving treatment that should have been treated was 15.2%. The total percentage of the population that would have self-cured was 62.1%, and overall treatment reduction was 32.2%. An ideal scenario for treatment decisions should be a scenario having high specificity and sensitivity and consequently lower false negative and false positive respectively. Additionally, the ability to select a test bases on sensitivity or specificity depends on the farm's needs if the farmer selects a test having higher sensitivity and lower specificity as we obtained in our models the number of animals been predicted as non-spontaneously curing and they can and they can spontaneously cure will be lower which is good for antibiotic usage reduction. However, a model with lower specificity will point out many animals as being able to spontaneously cure and they will fail to cure, which can be a problem considering animal welfare and the fact of metritis non-well resolution is associated with impaired reproductive performance, decreased milk production and high likelihood of culling (Oliveira et al. 2020).

## Conclusion

Overall, our multivariable logistic regression and ROC analysis indicate that cows developing metritis after the first week postpartum, having an increase in milk production starting at the day before metritis diagnosis day, and with a rectal temperature ≤ 39.3$^{o}$C at the time of metritis diagnosis have an increased odds of spontaneous cure of metritis, with an accuracy of 66% and sensitivity of 98%. Our machine learning algorithm achieved 70% accuracy for the prediction of SMC, and required only the variables for days in milk of metritis diagnosis and fever on the algorithm. Improving spontaneous metritis cure prediction is an important way to contribute toward antimicrobial stewardship due to promoting selective treatment for metritis, reducing the use of antibiotics, potentially reducing the dissemination of antimicrobial resistance, reducing the cost of the disease, and improving animal welfare.

Table 2-1. Descriptive statistics of cured and non-cured groups used in logistic regression and machine learning analysis.

| Item | MSC | | *P* |
|---|---|---|---|
| | YES | NO | |
| Number of animals enrolled (%) | 283 (64.6) | 155 (35.4) | |
| Number of primiparous animals enrolled (%) | 144 (64.8) | 78 (35.2) | 0.50 |
| Number of multiparous animals enrolled (%) | 139 (64.3) | 77 (35.7) | |
| Number of dystocia (%) | 64 (26.8) | 48 (30.1) | 0.06 |
| Number of animals with RFM (%) | 86 (30.3) | 49 (31.6) | 0.79 |
| Number of animals with vulvovaginal laceration (%) | 93 (32.8) | 60 (38.7) | 0.11 |
| Mean milk production slope 5 DIM[1] | 5.72 | 5.15 | 0.48 |
| Mean milk difference (Kg)[2] | 0.96 | -1.09 | <0.01 |
| Mean body condition score at 5 DIM | 3.33 | 3.35 | 0.59 |
| Mean DIM at metritis diagnosis | 6.3 | 7.3 | <0.01 |
| Mean rectal temperature at enrollment (ºC) | 39.2 | 39.4 | <0.01 |

[1] Mean milk production slope 5 DIM: Mean of milk production slope up to 5 DIM

[2] Mean milk difference (Kg): Mean milk production from the day before to the metritis diagnosis continuous.

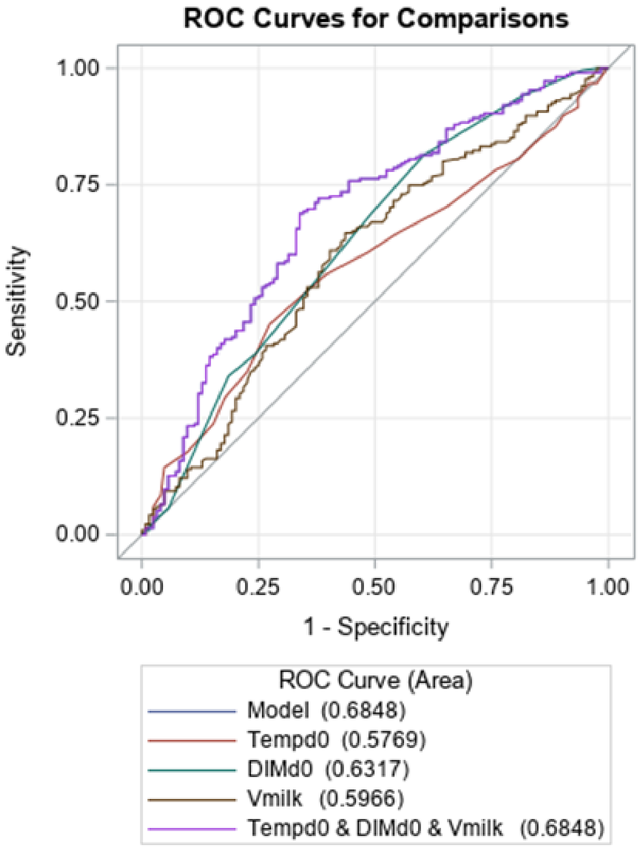


Figure 2-1. Optimal threshold values for the whole model. Tempd0: Rectal temperature at metritis diagnosis; DIMd0: Days in milk at metritis diagnosis; Vmilk: Milk production difference from day before to metritis diagnosis day.

Table 2-2. Outcomes from the final multivariable logistic regression model evaluating the association between cow related factors and spontaneous metritis cure.

| Variable | Level | Odds ratio (95% CI) | *P* |
|---|---|---|---|
| Milk difference Kg[1] | 0.91 Kg | *Baseline* | < 0.01 |
| | 1.91 Kg | 1.05 (1.01 – 1.09) | |
| Temperature | 39.3 ºC | *Baseline* | 0.02 |
| | 40.3 ºC | 0.63 (0.41 – 0.98) | |
| Dystocia | Yes | *Baseline* | 0.20 |
| | No | 1.42 (0.82 – 2.44) | |
| Days in milk (DO) | 7 days | *Baseline* | < 0.01 |
| | 8 days | 1.24 (1.09 – 1.41) | |

[1] Milk difference: Milk difference in Kg from the day before to the day of metritis diagnosis

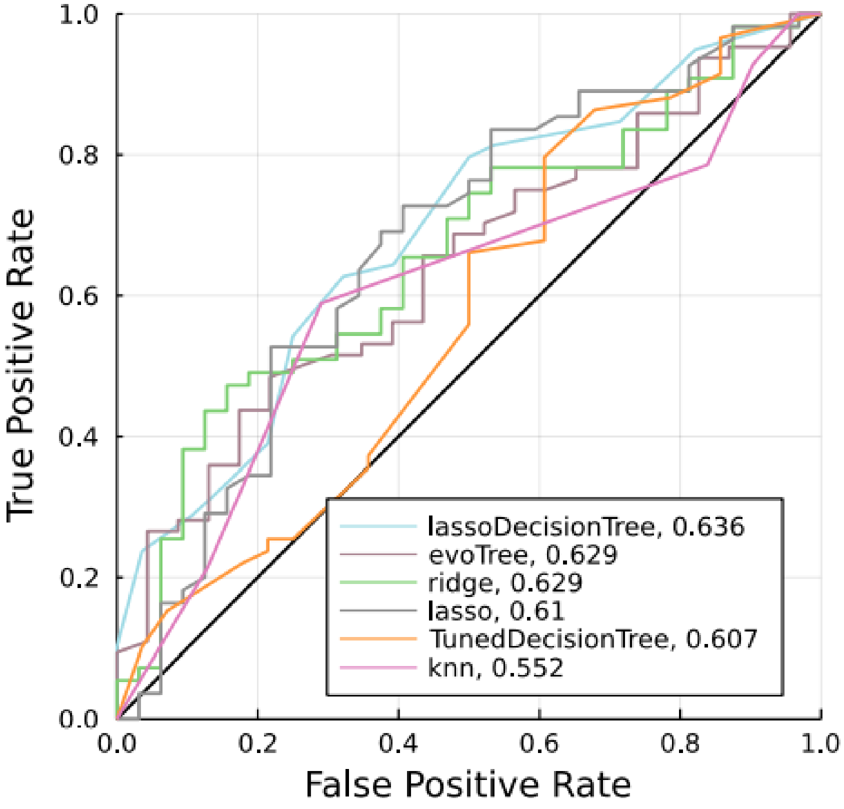


Figure 2-2. ROC curve for different classifiers trained on machine learning. The variables used for 5-fold cross-validation were rectal temperature at the metritis diagnosis day and days in milk of metritis diagnosis.

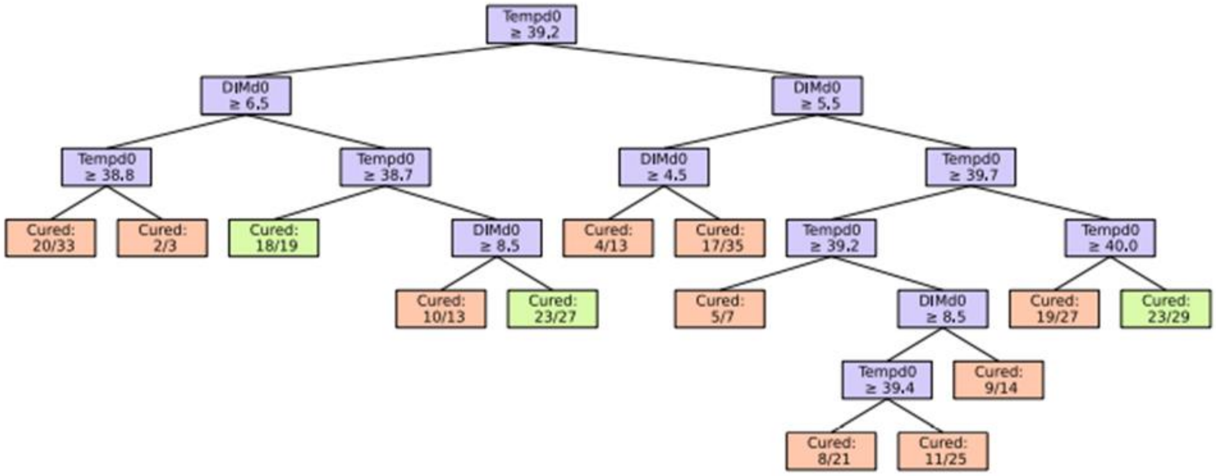


Figure 2-3. Decision tree analysis using different thresholds of rectal temperature at the metritis diagnosis day and days in milk of metritis diagnosis on spontaneous metritis cure. The leaf node color corresponds to whether the animal needs treatment, with red needing treatment, and green self-curing, considering a threshold for not treating 20% of animals that should receive antibiotic treatment.

## CHAPTER 3

# CHANGES IN THE UTERINE METABOLOME ASSOCIATED WITH METRITIS DEVELOPMENT AND CURE IN LACTATING HOLSTEIN COWS

### Abstract

The objective of this study was to characterize the changes in the uterine metabolome in cows with metritis development and cure for cows treated with antimicrobials due to metritis. This prospective cohort study was conducted in two dairies located in CA and TX. Vaginal discharge was evaluated and collected using the Metricheck® device. Cows were examined for metritis at 4, 7, and 9 DIM. Cows with fetid, watery, and reddish-brown uterine discharge were classified as having metritis and randomized to receive ceftiofur (n =10) or remain untreated (n = 7). The cure was defined as an absence of fetid, watery, reddish-brown uterine discharge at 14 d after enrollment. Vaginal discharge samples were collected from 86 cows within 6 hours after parturition, at 4 and 7 DIM, at metritis diagnosis, and at 4 and 7 days after metritis diagnosis. Cows with metritis (MET; n = 17) were paired with counterparts without metritis (NoMET) of similar DIM and parity (n = 49). The uterine metabolome was evaluated using untargeted gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). Metabolomic data were analyzed using the MetaboAnalyst software. Data were log-transformed and auto-scaled for normalization. Univariate analyses were performed, including fold change, principal component analysis, and partial least squares discriminant analysis. Comparing NoMET with MET cows at calving, 12 metabolites were upregulated, and 1 was downregulated ($P < 0.05$). The number of significantly different metabolites ($P < 0.05$) at 4 and 7 DIM were 51 and 74, respectively. After metritis development, three and five metabolites were upregulated in cows that were cured and in cows that received treatment and cured, respectively. In all scenarios, the metabolites lignoceric, malic, and maleic acids, ornithine, and hypotaurine, which are associated with arginine/aminoacyl-tRNA biosynthesis and taurine/purine metabolism, were upregulated in NoMET cows. This study demonstrates that metritis was associated with changes in the uterine metabolome. Also, cows not curing metritis had significant changes in the uterus metabolome independent of receiving ceftiofur or remaining untreated. Metabolome analysis may be an important tool helping to understand the uterus changes during the postpartum and the dynamic of metritis development.

**Key Words:** metabolomics, uterine health, metritis, maleic acid, ornithine

## Introduction

Metritis is a painful uterine pathology (Stojkov et al., 2015) that involves inflammation of all uterine layers (endometrium, myometrium, and serosa), and it is characterized by an abnormally enlarged uterus and a fetid, watery, red-brown uterine discharge within 21 days after parturition, with incidence peaking within the first ten days post-partum (Galvao, 2012). It is a postpartum disease with high incidence in dairy cows (Pinedo et al., 2020), and it has marked welfare, health, production, and reproduction (Pinedo et al., 2020; Oliveira et al., 2020; Lima et al., 2014), and economic consequences for the individual animal and the herd (Silva et al., 2021; Lima et al., 2019). The disease is associated with signs of systemic illness (decreased milk yield, dullness, or other signs of toxemia), and approximately half of the metritic cows can have fever (rectal temperature (RT) ≥ 39.5°C) (Benzaquen et al., 2007).

Metritis is a complex multifactorial disease caused by a mixed bacterial infection. Dairy cows unquestionably have bacterial contamination of the uterus in the first days following calving (Sheldon and Dobson., 2004a; Jeon et al., 2015). Previous studies showed that the microbiota is identical between cows that develop metritis and healthy cows up until two days postpartum (Jeon et al., 2015), but then the uterine microbiota diverges and dysbiosis of the uterine microbiota characterized by a loss of heterogeneity, a decrease in bacterial richness, an increase in Bacteroidetes and Fusobacteria, particularly Bacteroides, Porphyromonas, and Fusobacterium while the relative abundance of Proteobacteria and Tenericutes decrease (Galvao et al., 2019a). This suggests an association between the increase of those bacteria and metritis establishment. Additionally, recent data from uterine microbiome studies suggest that cows presenting risk factors for metritis (e.g., dystocia, stillbirth, and retained fetal membranes) are more likely to have uterine microbiota shifting towards pathogenic bacteria than herdmates free of these risk factors (Bicalho et al., 2010; Giuliodori et al., 2013).

During the transition into lactation, feed intake cannot meet the nutrient demands of high-producing dairy cows, resulting in a sharp decrease in glucose and minerals (Drackley et al., 1999; Bell 1995). Cows enter in negative energy balance leading to body fat mobilization in the form of non-esterified fatty acids (NEFA) and accumulation of products of incomplete oxidation of NEFA such as beta-hydroxybutyrate (BHB) (Ospina et al., 2010). The decrease in glucose and calcium concentrations and the increase in NEFA and BHB concentrations are associated with immunosuppression and increased risk of metritis. Dervishi et al. 2016, reported that cows with metritis show alterations in metabolites related to carbohydrate metabolism, acute phase proteins, and proinflammatory cytokines starting at eight and four weeks before parturition and the appearance of clinical signs of metritis. In addition, byproducts of bacterial metabolisms, such as proteins, short-chain fatty acids, and other metabolites, affect immune function (Contreras and Sordillo, 2011). In cows, the vascular degeneration that occurs shortly after calving allows blood to seep into the uterine lumen, which allows for the exchange of metabolites between blood and the uterus (Jeon et al., 2017). This exchange means that blood metabolites can affect the uterine microbiota, whereas microbial-derived metabolites affect leukocytes in blood and tissues, which can affect bacterial proliferation in the uterus.

Many studies have reported the associations of individuals' minerals or blood metabolites with immune function and metritis development (Machado et al., 2020; Chapinal et al., 2011). However, studies characterizing the vaginal-uterine metabolome and showing how it affects the mucosal immune system of the uterus and the risk of metritis are still scarce. Analytical approaches such as metabolomics technology are advancing and refer to the analysis of concentration changes in small molecule metabolites after organisms experience temporal and external stimuli (Wang et al., 2021). The primary analytical approaches used in metabolomics rely on two techniques: nuclear magnetic resonance (NMR) and mass spectrometry (MS) (Gowda and Djukovic, 2013). NMR determines the magnetic resonance of nuclei in the molecule and is suitable for detecting all compounds that contain hydrogen atoms. At the same time, MS detection is the most frequently used platform in metabolomics quantification. The technology of metabolomics improved the precision of identifying and quantifying low molecular weight metabolites and their intermediates in different biofluids or tissues which makes possible a better understanding of other diseases processes in dairy animals and the application of this technology has been reported in studies focusing on ketosis (Zhang et al., 2013; Sun et al. 2014), milk fever, subclinical mastitis and urine fingerprinting for metritis risk prediction (Hailemariam et al. 2014a, b; Dervishi et al. 2017, Dervishi et al. 2018).

Characterizing vaginal-uterine metabolome during the peripartum postpartum can help unravel the crosstalk between host immunological factors and microbes that are key for metritis development and cure. In this sense, the objectives of this study were to characterize the changes in the uterine metabolome in Holstein cows associated with metritis development, cure, and antimicrobial treatment using untargeted gas chromatography time-of-flight mass spectrometry. Our premise is that metritis development and cure are associated with changes in uterine metabolome after calving, and that antimicrobial treatment is associated with uterine metabolome changes in metritic cows.

## Materials and Methods

### Data and Animals

This prospective cohort study was conducted in two dairies in the central valley, California (4,300 lactating Holstein cows) and one dairy in northwest Texas (2,900 lactating Holstein cows) from August to October 2020, and January 2020 to May 2021, respectively. All herds milked only Holstein cows. The rolling herd's average milk yield ranged from 10,150 to 12,000 kg. Postpartum pens had sand-bedded stalls and were equipped with sprinklers over the feeding areas activated when the environmental temperature rose above > 21 °C. The postpartum diet was formulated to meet or exceed the dietary nutrient requirements for a lactating cow weighing 680 kg and producing 45 kg of 3.5% fat-corrected milk and 3.0% protein (NRC, 2001), and it was delivered as a TMR twice daily.

Vaginal discharge samples were collected from 86 cows within 6 hours after parturition, at 4 and 7 DIM, at metritis diagnosis, and at 4 and 7 days after metritis diagnosis. All animals enrolled on the parturition day were assessed for metritis diagnosis at 5, 7, and 9 days in milk (DIM), and vaginal discharge was evaluated using the Metricheck® device (Simcro, Hamilton, NZ). Discharge retrieved from the vagina was scored as 1 = not fetid normal lochia, viscous, clear, red, or brown; 2 = cloudy mucoid discharge with flecks of pus; 3 = not fetid, mucopurulent discharge with < 50% pus; 4 = not fetid mucopurulent white, yellow or reddish-brownish discharge with ≥ 50% pus; and, 5= fetid, thin, serous, or watery, may have been reddish-brownish, with or without pieces of necrotic tissue present (adapted from Chenault et al., 2004). Cows with fetid, watery, and reddish-brownish discharge, with or without pieces of necrotic tissue present, were classified as having metritis (Sheldon et al., 2006). Metritic cows were randomly assigned to one of two treatments: 1- Ceftiofur [(n = 10) = subcutaneous injections of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) in the base of the ear at D0 and D3. Live body weight was estimated using a heart girth measuring tape (Nasco Inc., Atkinson, WI)]; 2 – CON [(n = 7) = remained untreated at the time of metritis diagnosis]. For vaginal discharge metabolomic analysis, cows with metritis (MET; n = 17) were paired with counterparts without metritis (NoMet) of similar DIM and parity (n = 49). Cows with a vaginal discharge ≤ 3 were classified as NoMet.  Cows previously diagnosed with metritis with vaginal discharge score < 5 on d 14 after enrollment were considered cured. The post-enrollment exam on d 14 was performed by a veterinarian from the research team that was unaware of the treatment assignment.

### Metabolomics Data Acquisition and Processing

Vaginal discharges were collected using the Metricheck® device, transferred to two polypropylene vials and stored at -80 °C until analyzed in an untargeted gas chromatography time-of-flight mass spectrometer (GC-TOF-MS) at UC – Davis West Coast Metabolomics Center. The retention index and the complete mass spectrum were encoded as a string. All thresholds reflect settings for ChromaTOF v. 2.32. Quantification was reported as peak height using the unique ion as the default unless a different quantification ion was manually set in the BinBase administration software BinView. We detected 174 known metabolites from 368 untargeted primary metabolites found in our analysis. A column of 30 m length by 0.25 mm internal diameter with 0.25 μm film made of 95% dimethyl/5diphenyl polysiloxanes was used in a Restek corporation Rtx-5Sil MS. The gas helium (99.99% purity) was used a carrier for the analysis. The column temperature was set between 50 – 330˚C at flow-rate of 1 mL min-1. The oven temperature was set to 50˚C for 1 min, then ramped at 20˚C min-1 to 330˚C, and held constant for 5 min. Finally, the injection temperature was set to 50˚C and ramped to 250˚C by increments of 12˚C-1. The retention of primary metabolites (amino acids, hydroxyl acids, carbohydrates, sugar acids, sterols, aromatics, nucleosides, amines, and various compounds) was evaluated.

### Metabolomic Statistical Analysis

Metabolomic analyses were performed using Metaboanalyst 5.0 (www.metaboanalyst.ca). Before data analysis, a data filtering and integrity check were performed to ensure that all the necessary information was collected (two classes, nonnegative numbers for compound concentration or peak intensity values, and missing values imputations). Data were log-transformed (base 10) and auto-scaled for normalization. For explanatory data analysis, a univariate analysis was performed. To identify candidate metabolites linked with the metritis development and cure, a fold change analysis (FC) was performed with a threshold of 2. To select the essential features based on biological and statistical significance, a volcano plot analysis was performed using fold change threshold (x) 2 and t-tests threshold (y) 0.1. Principal component analysis (PCA), partial-least square discriminant analysis (PLS-DA), and Orthogonal PLS-DA analyses were performed to understand metabolite differences between animals developing or not metritis and curing or not from metritis. Pathways of different metabolites for models using two different organisms (cow (Bos taurus) and E. coli) were further screened using enrichment and topological analyses to identify the key pathway most highly correlated with metabolite differences.

## Results

### Number of cows enrolled per farm and descriptive data

Overall, 86 animals were enrolled, 44 in California and 42 in Texas. The number of animals developing metritis were 9 and 8 at the dairy in California and Texas, respectively. Average lactation was 2.4. The mean days in milk at metritis diagnosis was 6 days.  and mean BCS of animals enrolled and developing metritis were 3.23 and 3.5 respectively.

### Changes in uterine metabolome in cows developing metritis

A total of 185 known and 236 unknown primary metabolites were identified in the vaginal discharge. At calving, fold change analysis revealed 38 metabolites (32 up-regulated and six down-regulated). Within six hours after calving, comparing [(NoMET vs.

MET (animals that developed metritis later)], 12 metabolites were up-regulated (maltose, nicotinamide, maltotriose, 1,5-anhydroglucitol, panose, glucose, adenosine, linoleic acid, alpha-aminoadipic, taurine, hypotaurine, and inosine) and one metabolite was downregulated (thymine) ($P < 0.05$) Table 1. PLS-DA with known metabolites indicated a slight dispersion in metabolites profile within health and metritic cows at calving day (Figure 1A, B). At 4 DIM, fold change analysis revealed 51 essential metabolites, and comparing NoMET to MET groups, 38 metabolites were up-regulated, and 13 metabolites were down-regulated. The top 50 metabolites are presented in table 2. Overall, PLS-DA with known metabolites indicated an increased dispersion on metabolites profile within health and metritic cows at calving day (Figure 2 A, B). At 7 DIM, fold change analysis revealed 74 important metabolites, and comparing NoMET to MET groups, 49 metabolites were up-regulated, and 22 metabolites were down-regulated. The top 50 metabolites are presented in table 3. Overall, PLS-DA with known metabolites indicated a greater dispersion on metabolites profile within health and metritic cows at 7 DIM compared to calving day and 4 DIM (Figure 3 A, B). Pathway analysis based on identified metabolites in cows not developing compared to cows developing metritis allowed the understanding between the biological pathways for cows (Bos Taurus) and *Escherichia coli*. The subset of important metabolites is associated with glutathione, taurine, hypotaurine, alanine, aspartate, glutamate, D-glutamine, D-glutamate and phenylalanine metabolism, and aminoacyl-tRNA biosynthesis in Bos Taurus organisms and associated with glutathione metabolism and aminoacyl-tRNA biosynthesis.

### Changes in uterine metabolome in cows curing vs non-curing from metritis

A total of 185 known and 236 unknown primary metabolites were identified in the vaginal discharge. After metritis development, three metabolites (lignoceric acid, maleic acid, and ornithine) were upregulated ($P < 0.05$) in cows curing compared to non-curing cows from metritis and receiving or not treatment. For the group of animals that received treatment and cured, five metabolites (lignoceric acid, maleic acid, malic acid, N-acetylmannosamine, and ornithine) were upregulated ($P < 0.05$) in cows receiving treatment and curing compared to cows receiving treatment and not curing from metritis. Overall, PLS-DA with known metabolites indicated a dispersion on metabolites profile in cows curing (treated and untreated) and in cows that received treatment and cured, in figures 4 (A, B) and 5 (A, B), respectively. Pathway analysis based on identified metabolites in cows curing from metritis allowed the understanding between the biological pathways for cows (Bos Taurus) and E. Coli. In both scenarios, the subset of essential metabolites is associated with arginine, proline, glyoxylate, dicarboxylate and phenylalanine metabolism, and aminoacyl-tRNA biosynthesis in Bos Taurus organisms and associated with arginine and proline metabolism, and arginine biosynthesis in E. Coli organisms.

## Discussion

The current study shows significant changes in the uterine metabolome in cows developing and curing metritis. Changes in metabolic pathways during the transition period, such as amino acids conversion to glucose and increased blood metabolites such as NEFA and BHB that are associated with dairy cows' health and metritis have been intensively studied (Ospina et al., 2010; Reynolds et al., 2013; Luo et al., 2019). On the other hand, studies evaluating metabolomic changes in the uterus during the first weeks postpartum and its association with immunity and metritis are still scarce. Our objectives in performing this study were to characterize the changes in the uterine metabolome in

Holstein cows associated with metritis development, cure, and antimicrobial treatment using untargeted gas chromatography time-of-flight mass spectrometry.

An interesting finding of this study was that the metabolome profile in the uterus changed from a few hours after calving up to the day of the metritis event, and the number of metabolites up and downregulated increased throughout the time, which represents an increase in metabolites difference up to 7 DIM within cows developing and not developing metritis. The leading metabolites up and downregulated were sugar (e.g., glucose, maltose, panose), and amino acids (adenosine, taurine, and hypotaurine) derived metabolites, and other important organic compounds such as nicotinamide and linoleic acid. Sheldon et al. (2020) suggested that preventing uterine disease in dairy cattle depends on avoiding, tolerating, and resisting pathogenic bacteria. In other words, it depends on the ability to mitigate tissue damage caused by pathogens through neutralizing toxins, tissue repair, and immune competence. Those important metabolites are known to have an essential role in immune response and may represent the changes in uterus dysbiosis, toxins' neutralization, and tissue repair.

The main pathways related to the metabolite changes in the uterus associated with the Bos taurus at an organism level are taurine, hypotaurine, purine, glutathione, and phenylalanine metabolisms which have a positive association with immunity and health. Nicotinamide concentration was increased in cows not developing metritis, suggesting that this metabolite regulates important physiologic processes in the uterus. Nicotinamide is a critical regulator for maintaining important physiologic functions and acting on infection and inflammation (Xie et al., 2020). Griffiths et al. 2020, reported the essential redox effect of nicotinamide in promoting cellular oxidative (catabolic) metabolic disorders and as an anti-cancer and anti-aging therapeutic target. Other metabolites being upregulated in cows not developing metritis are taurine and hypotaurine; both are antioxidant sulfur-containing amino acids that improve immune function due to anti-apoptotic activities, antioxidant stress effects, and regulation of mitochondrial function (Jong et al., 2012; Marcinkiewicz et al., 2013). All positive effects associated with these metabolites can be associated with improved immune response, the rapid ability of the uterus tissue to respond to damage, and consequently reduced chances of pathogenic bacteria growth.

Cows have an established uterine microbiota within 20 minutes of calving (Jeon et al., 2015). The microbiota structure of a high proportion of proteobacteria is identical between cows that develop metritis and healthy cows up until two days post-partum, after which the bacterial community structure deviates in favor or greater relative abundance of Bacteroides, Porphyromonas, and Fusobacterium in metritic cows (Jeon et al., 2015; Jeon et al., 2017). Regarding the pathways associated with *E. coli* in the first week post-partum, it was mainly associated with specifics pathway of glutathione, taurine, and hypotaurine metabolism and aminoacyl-tRNA biosynthesis, which are directly associated with protein biosynthesis, which induces translation and *E. coli* growth (Putzer et al., 1995). Glutathione, taurine, and hypotaurine metabolisms pathway are associated with preventing inflammation and blocking bacterial growth, which would alleviate bacterial dysbiosis (Yu et al., 2016). Metabolite changes reported are likely related to reduced metritis development in the uterus.

Cows not curing metritis had significant changes in the uterus metabolome independent of receiving ceftiofur or remaining untreated. Comparing cured to uncured animals, animals curing had a higher concentration of lignoceric, malic, maleic, and lauric acids and ornithine. The main pathways related to the metabolites changes in the uterus associated with the *Bos taurus* at an organism level are Aminoacyl-TRNA biosynthesis, glutathione, taurine, and hypotaurine metabolism, which match with the pathways associated with cows that did not develop metritis and suggest that these pathways are associated with immunity, uterus integrity, and pathogenic bacteria reduction which may reduce the severity of the disease and increases healing from metritis. The main pathways related to the metabolite changes in the uterus associated with the *E. coli* at organism levels are arginine biosynthesis and arginine and proline metabolism. Those pathways were reported as associated with gut microecology and *E. coli* reduction (Yu et al., 2016).

Additionally, some studies highlighted possible connections between L-arginine and Bacteroides spp. and found that increased arginine concentration correlated to reduced Bacteroides growth. Metritic cows had a greater abundance of total bacteria and a greater abundance of *Bacteroides pyogenes (B. pyogenes)*, *Porphyromonas levii (P. levii)*, and *Fusobacterium necrophorum (F. necrophorum)* (Jeon et al., 2017). However, suppose the metabolites in the uterus of metritis, even after disease establishment, can alter pathogenic growth and reduce dysbiosis. In that case, it could minimize the severity and increase curing independent of treatment, as shown in our results.

One limitation of this study is that we collected vaginal discharge for the metabolome analysis. However, it’s known that unless a cow has severe vaginitis after calving, the discharge in the vagina is mainly from the uterus due to the uterus cleanness and involution. Therefore, vaginal discharge represents very well the uterus discharge.

Additionally, the Metricheck tool has been compared with other standard methods for evaluation of vaginal discharge for the diagnosis of uterine disease, such as cytology, manual evaluation, and vaginoscopy, and it was found to have higher sensitivity than vaginoscopy for the diagnosis of uterine disease such endometritis (McDougall et al., 2007; Pleticha et al., 2009). Further studies should focus on increasing the sample size of animals with metritis and assessing the metabolites associated with spontaneous cure. In this study we could not analyze metabolites changes in cows self-curing from metritis due to the limited number of animals left untreated and curing. Additionally, performing studies using different methods for discharge collection and comparing how the metabolome from plasma and uterine fluid is associated will be essential to determine what metabolites are related to immune function and pathogenic bacterial proliferation in the uterus. Those approaches would be beneficial to identify biomarkers associated with improved immune function, reduced pathogenic bacterial growth in the uterus, and improved uterine health.

## Conclusion

In summary, untargeted GC-TOF-MS metabolomic analysis of vaginal discharge of cows in the first week postpartum, at metritis diagnosis, and at 4 and 7 days after metritis diagnosis, highlighted changes in the uterine metabolome in the first week postpartum in cows developing metritis comparing to healthy animals. For the metritic group there are significant changes in the uterine metabolome associated to cure. In all scenarios, the metabolites lignoceric, malic, and maleic acids, ornithine, and hypotaurine, which are associated with arginine/aminoacyl-tRNA biosynthesis and taurine/purine metabolism, were upregulated in NoMET group and in cows curing from metritis. Also, cows not curing from metritis had significant changes in the uterus metabolome independent of receiving ceftiofur or remaining untreated. Metabolome analysis may be an important tool to understand changes in the uterus during the postpartum and the dynamic of metritis development.

Table 3-1. Important metabolites that are up and down regulated within 6 hours postpartum in healthy cows compared to animals developing metritis.

| | Compounds | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|---|
| 1 | maltose | 8.2008 | 3.0358 | 0.001909 | 2.7192 |
| 2 | nicotinamide | 2.2286 | 1.1562 | 0.0027633 | 2.5586 |
| 3 | maltotriose | 8.2341 | 3.0416 | 0.0072505 | 2.1396 |
| 4 | 1,5-anhydroglucitol | 2.1199 | 1.084 | 0.012228 | 1.9127 |
| 5 | panose | 6.5977 | 2.722 | 0.013923 | 1.8563 |
| 6 | glucose | 4.0647 | 2.0231 | 0.016681 | 1.7778 |
| 7 | adenosine | 2.0373 | 1.0266 | 0.021417 | 1.6692 |
| 8 | linoleic acid | 2.6882 | 1.4266 | 0.02161 | 1.6653 |
| 9 | alpha-aminoadipic acid | 3.9039 | 1.9649 | 0.022083 | 1.6559 |
| 10 | taurine | 2.6779 | 1.4211 | 0.026722 | 1.5731 |
| 11 | hypotaurine | 2.0738 | 1.0523 | 0.055062 | 1.2591 |
| 12 | inosine | 2.5176 | 1.332 | 0.078835 | 1.1033 |
| 13 | thymine | 0.38734 | -1.3683 | 0.080836 | 1.0924 |

Important features selected by volcano plot with fold change (FC) threshold (x) 2 and t-tests threshold (y) 0.1. Both fold changes and p values are log transformed. The further its position away from the (0,0), the more significant the feature is.

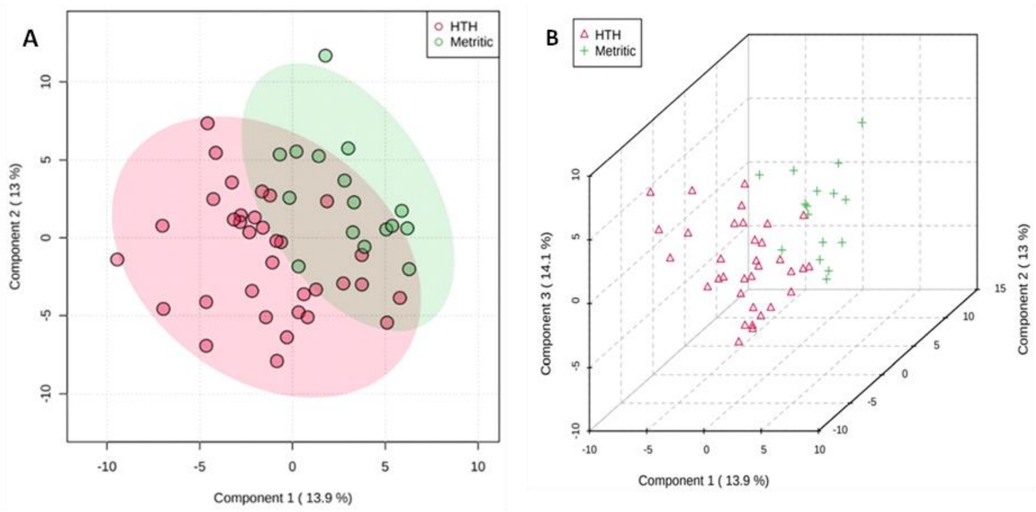
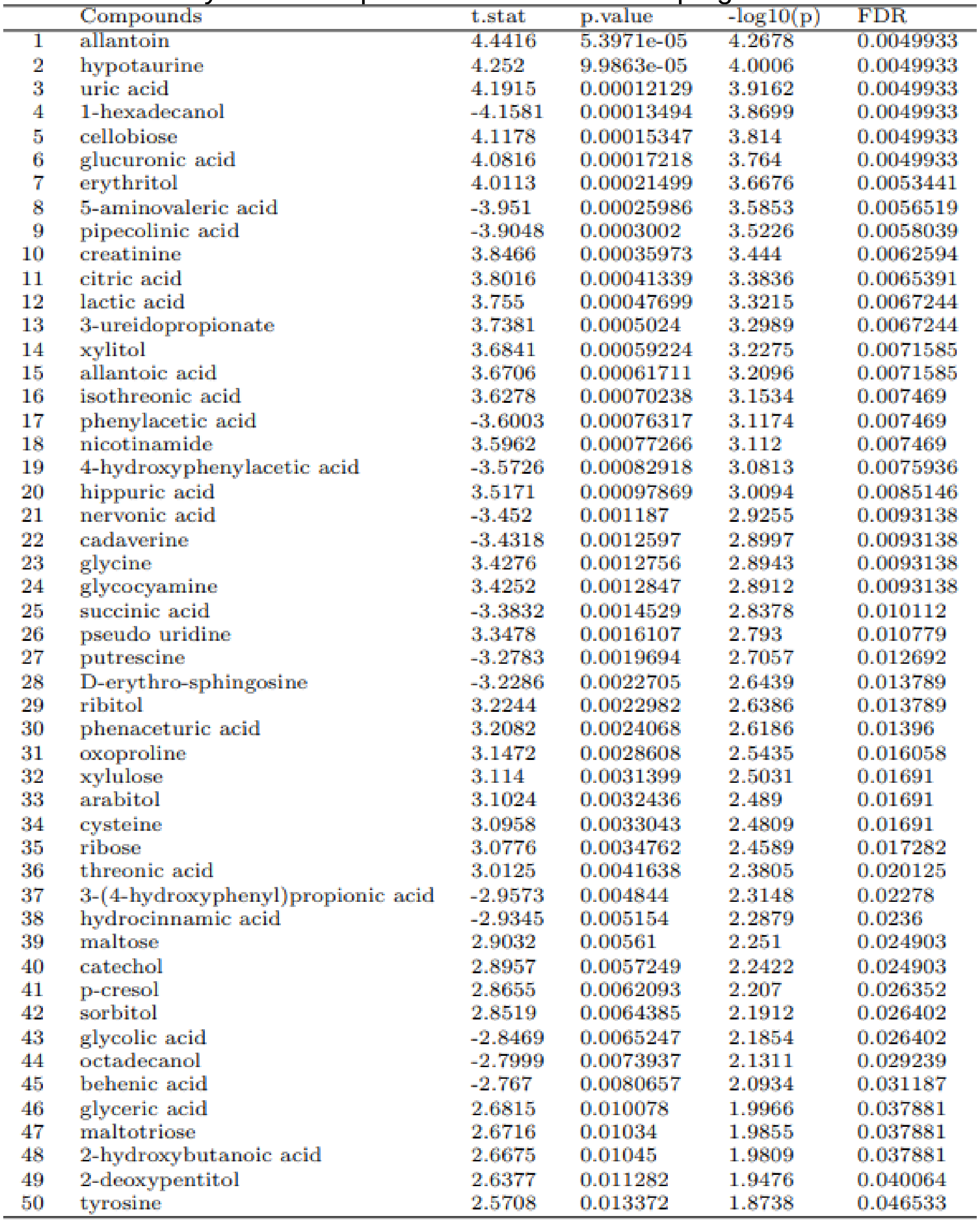


Figure 3-1. Partial least square-discriminant analysis (PLS-DA) of vaginal-uterine discharge metabolites within 6 hours post-partum in healthy cows compared to animals developing metritis. Scores plot between the selected principal components; the explained variances are shown in brackets (A). 3-D representation of known (B) metabolome composition is displayed to demonstrate that there is a slight metabolome difference within 6 hours post-partum between cows developing or not metritis.

Table 3-2. Important metabolites that are up and down regulated within at 4 DIM in healthy cows compared to animals developing metritis.



| | Compounds | t.stat | p.value | -log10(p) | FDR |
|---|---|---|---|---|---|
| 1 | allantoin | 4.4416 | 5.3971e-05 | 4.2678 | 0.0049933 |
| 2 | hypotaurine | 4.252 | 9.9863e-05 | 4.0006 | 0.0049933 |
| 3 | uric acid | 4.1915 | 0.00012129 | 3.9162 | 0.0049933 |
| 4 | 1-hexadecanol | -4.1581 | 0.00013494 | 3.8699 | 0.0049933 |
| 5 | cellobiose | 4.1178 | 0.00015347 | 3.814 | 0.0049933 |
| 6 | glucuronic acid | 4.0816 | 0.00017218 | 3.764 | 0.0049933 |
| 7 | erythritol | 4.0113 | 0.00021499 | 3.6676 | 0.0053441 |
| 8 | 5-aminovaleric acid | -3.951 | 0.00025986 | 3.5853 | 0.0056519 |
| 9 | pipecolinic acid | -3.9048 | 0.0003002 | 3.5226 | 0.0058039 |
| 10 | creatinine | 3.8466 | 0.00035973 | 3.444 | 0.0062594 |
| 11 | citric acid | 3.8016 | 0.00041339 | 3.3836 | 0.0065391 |
| 12 | lactic acid | 3.755 | 0.00047699 | 3.3215 | 0.0067244 |
| 13 | 3-ureidopropionate | 3.7381 | 0.0005024 | 3.2989 | 0.0067244 |
| 14 | xylitol | 3.6841 | 0.00059224 | 3.2275 | 0.0071585 |
| 15 | allantoic acid | 3.6706 | 0.00061711 | 3.2096 | 0.0071585 |
| 16 | isothreonic acid | 3.6278 | 0.00070238 | 3.1534 | 0.007469 |
| 17 | phenylacetic acid | -3.6003 | 0.00076317 | 3.1174 | 0.007469 |
| 18 | nicotinamide | 3.5962 | 0.00077266 | 3.112 | 0.007469 |
| 19 | 4-hydroxyphenylacetic acid | -3.5726 | 0.00082918 | 3.0813 | 0.0075936 |
| 20 | hippuric acid | 3.5171 | 0.00097869 | 3.0094 | 0.0085146 |
| 21 | nervonic acid | -3.452 | 0.001187 | 2.9255 | 0.0093138 |
| 22 | cadaverine | -3.4318 | 0.0012597 | 2.8997 | 0.0093138 |
| 23 | glycine | 3.4276 | 0.0012756 | 2.8943 | 0.0093138 |
| 24 | glycocyamine | 3.4252 | 0.0012847 | 2.8912 | 0.0093138 |
| 25 | succinic acid | -3.3832 | 0.0014529 | 2.8378 | 0.010112 |
| 26 | pseudo uridine | 3.3478 | 0.0016107 | 2.793 | 0.010779 |
| 27 | putrescine | -3.2783 | 0.0019694 | 2.7057 | 0.012692 |
| 28 | D-erythro-sphingosine | -3.2286 | 0.0022705 | 2.6439 | 0.013789 |
| 29 | ribitol | 3.2244 | 0.0022982 | 2.6386 | 0.013789 |
| 30 | phenaceturic acid | 3.2082 | 0.0024068 | 2.6186 | 0.01396 |
| 31 | oxoproline | 3.1472 | 0.0028608 | 2.5435 | 0.016058 |
| 32 | xylulose | 3.114 | 0.0031399 | 2.5031 | 0.01691 |
| 33 | arabitol | 3.1024 | 0.0032436 | 2.489 | 0.01691 |
| 34 | cysteine | 3.0958 | 0.0033043 | 2.4809 | 0.01691 |
| 35 | ribose | 3.0776 | 0.0034762 | 2.4589 | 0.017282 |
| 36 | threonic acid | 3.0125 | 0.0041638 | 2.3805 | 0.020125 |
| 37 | 3-(4-hydroxyphenyl)propionic acid | -2.9573 | 0.004844 | 2.3148 | 0.02278 |
| 38 | hydrocinnamic acid | -2.9345 | 0.005154 | 2.2879 | 0.0236 |
| 39 | maltose | 2.9032 | 0.00561 | 2.251 | 0.024903 |
| 40 | catechol | 2.8957 | 0.0057249 | 2.2422 | 0.024903 |
| 41 | p-cresol | 2.8655 | 0.0062093 | 2.207 | 0.026352 |
| 42 | sorbitol | 2.8519 | 0.0064385 | 2.1912 | 0.026402 |
| 43 | glycolic acid | -2.8469 | 0.0065247 | 2.1854 | 0.026402 |
| 44 | octadecanol | -2.7999 | 0.0073937 | 2.1311 | 0.029239 |
| 45 | behenic acid | -2.767 | 0.0080657 | 2.0934 | 0.031187 |
| 46 | glyceric acid | 2.6815 | 0.010078 | 1.9966 | 0.037881 |
| 47 | maltotriose | 2.6716 | 0.01034 | 1.9855 | 0.037881 |
| 48 | 2-hydroxybutanoic acid | 2.6675 | 0.01045 | 1.9809 | 0.037881 |
| 49 | 2-deoxypentitol | 2.6377 | 0.011282 | 1.9476 | 0.040064 |
| 50 | tyrosine | 2.5708 | 0.013372 | 1.8738 | 0.046533 |

Important features selected by volcano plot with fold change (FC) threshold (x) 2 and t-tests threshold (y) 0.1. Both fold changes and p values are log transformed. The further its position away from the (0,0), the more significant the feature is.

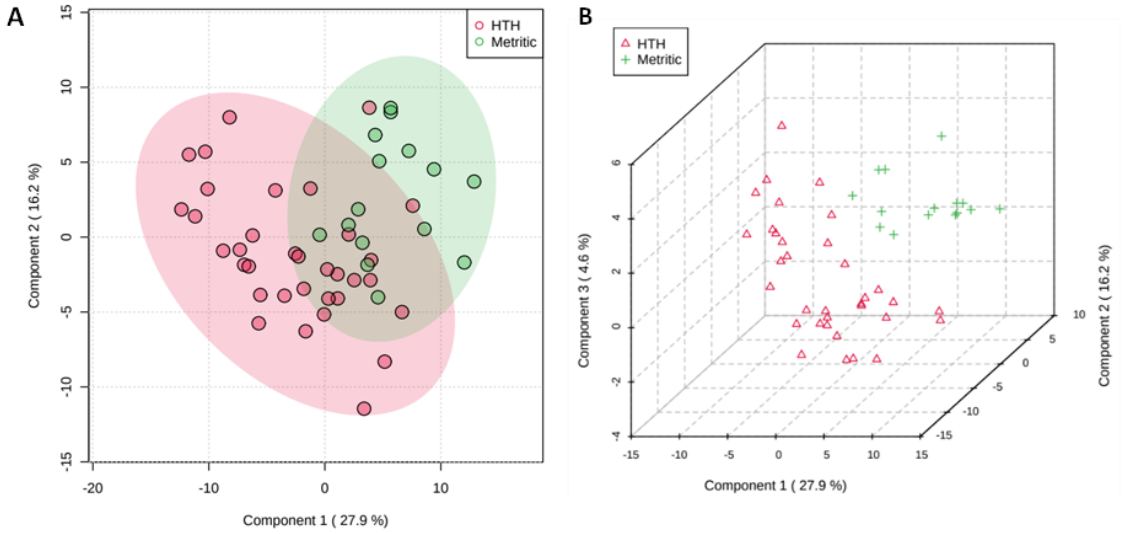
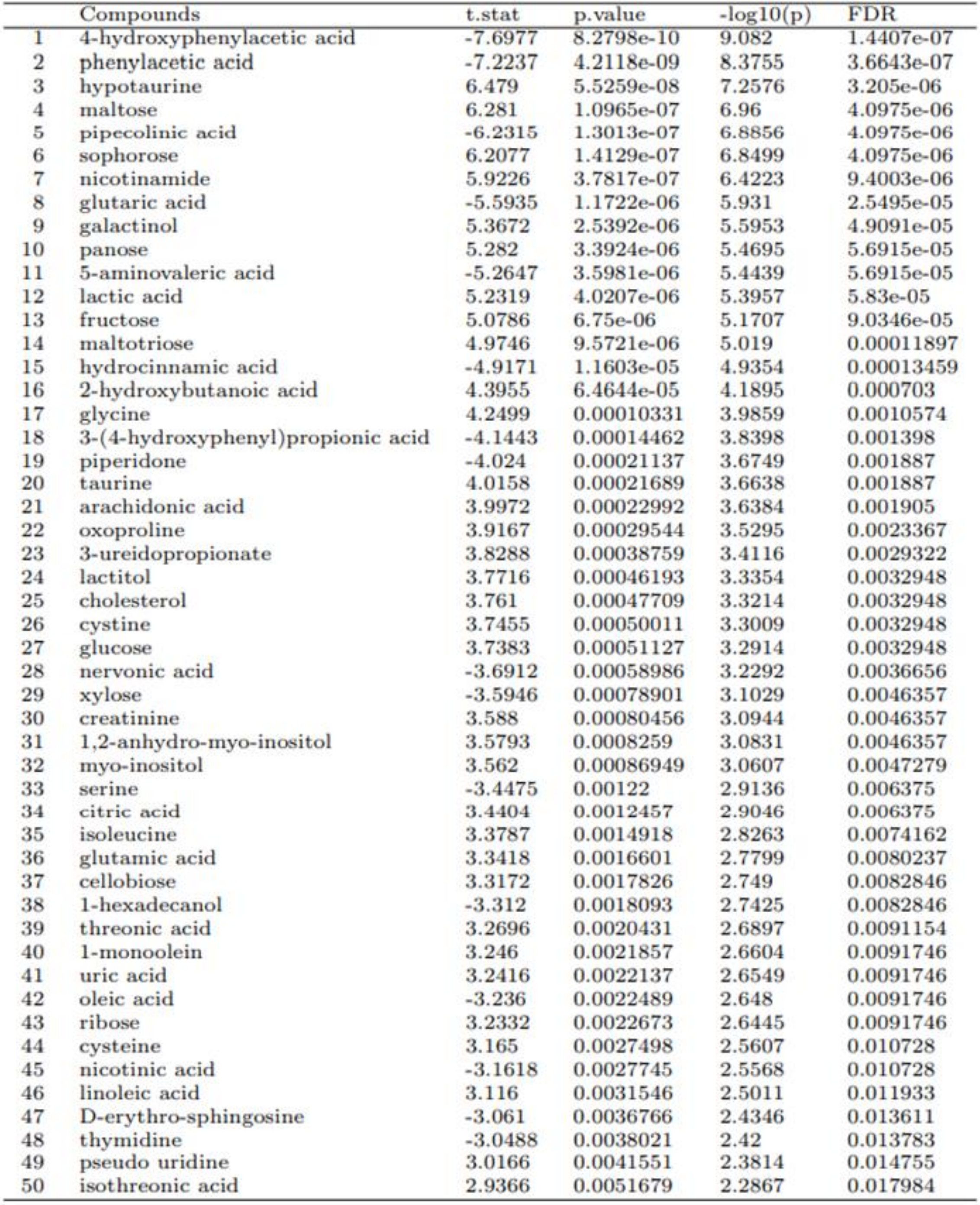


Figure 3-2. Partial least square-discriminant analysis (PLS-DA) of vaginal-uterine discharge metabolites at 4 DIM in healthy cows compared to animals developing metritis. Scores plot between the selected principal components; the explained variances are shown in brackets (A). 3-D representation of known (B) metabolome composition is displayed to demonstrate that there is a metabolome difference at 4 DIM between cows developing or not metritis.

Table 3-3. Important metabolites that are up and down regulated at 7 DIM in healthy cows compared to animals developing metritis.



|  | Compounds | t.stat | p.value | -log10(p) | FDR |
|---|---|---|---|---|---|
| 1 | 4-hydroxyphenylacetic acid | -7.6977 | 8.2798e-10 | 9.082 | 1.4407e-07 |
| 2 | phenylacetic acid | -7.2237 | 4.2118e-09 | 8.3755 | 3.6643e-07 |
| 3 | hypotaurine | 6.479 | 5.5259e-08 | 7.2576 | 3.205e-06 |
| 4 | maltose | 6.281 | 1.0965e-07 | 6.96 | 4.0975e-06 |
| 5 | pipecolinic acid | -6.2315 | 1.3013e-07 | 6.8856 | 4.0975e-06 |
| 6 | sophorose | 6.2077 | 1.4129e-07 | 6.8499 | 4.0975e-06 |
| 7 | nicotinamide | 5.9226 | 3.7817e-07 | 6.4223 | 9.4003e-06 |
| 8 | glutaric acid | -5.5935 | 1.1722e-06 | 5.931 | 2.5495e-05 |
| 9 | galactinol | 5.3672 | 2.5392e-06 | 5.5953 | 4.9091e-05 |
| 10 | panose | 5.282 | 3.3924e-06 | 5.4695 | 5.6915e-05 |
| 11 | 5-aminovaleric acid | -5.2647 | 3.5981e-06 | 5.4439 | 5.6915e-05 |
| 12 | lactic acid | 5.2319 | 4.0207e-06 | 5.3957 | 5.83e-05 |
| 13 | fructose | 5.0786 | 6.75e-06 | 5.1707 | 9.0346e-05 |
| 14 | maltotriose | 4.9746 | 9.5721e-06 | 5.019 | 0.00011897 |
| 15 | hydrocinnamic acid | -4.9171 | 1.1603e-05 | 4.9354 | 0.00013459 |
| 16 | 2-hydroxybutanoic acid | 4.3955 | 6.4644e-05 | 4.1895 | 0.000703 |
| 17 | glycine | 4.2499 | 0.00010331 | 3.9859 | 0.0010574 |
| 18 | 3-(4-hydroxyphenyl)propionic acid | -4.1443 | 0.00014462 | 3.8398 | 0.001398 |
| 19 | piperidone | -4.024 | 0.00021137 | 3.6749 | 0.001887 |
| 20 | taurine | 4.0158 | 0.00021689 | 3.6638 | 0.001887 |
| 21 | arachidonic acid | 3.9972 | 0.00022992 | 3.6384 | 0.001905 |
| 22 | oxoproline | 3.9167 | 0.00029544 | 3.5295 | 0.0023367 |
| 23 | 3-ureidopropionate | 3.8288 | 0.00038759 | 3.4116 | 0.0029322 |
| 24 | lactitol | 3.7716 | 0.00046193 | 3.3354 | 0.0032948 |
| 25 | cholesterol | 3.761 | 0.00047709 | 3.3214 | 0.0032948 |
| 26 | cystine | 3.7455 | 0.00050011 | 3.3009 | 0.0032948 |
| 27 | glucose | 3.7383 | 0.00051127 | 3.2914 | 0.0032948 |
| 28 | nervonic acid | -3.6912 | 0.00058986 | 3.2292 | 0.0036656 |
| 29 | xylose | -3.5946 | 0.00078901 | 3.1029 | 0.0046357 |
| 30 | creatinine | 3.588 | 0.00080456 | 3.0944 | 0.0046357 |
| 31 | 1,2-anhydro-myo-inositol | 3.5793 | 0.0008259 | 3.0831 | 0.0046357 |
| 32 | myo-inositol | 3.562 | 0.00086949 | 3.0607 | 0.0047279 |
| 33 | serine | -3.4475 | 0.00122 | 2.9136 | 0.006375 |
| 34 | citric acid | 3.4404 | 0.0012457 | 2.9046 | 0.006375 |
| 35 | isoleucine | 3.3787 | 0.0014918 | 2.8263 | 0.0074162 |
| 36 | glutamic acid | 3.3418 | 0.0016601 | 2.7799 | 0.0080237 |
| 37 | cellobiose | 3.3172 | 0.0017826 | 2.749 | 0.0082846 |
| 38 | 1-hexadecanol | -3.312 | 0.0018093 | 2.7425 | 0.0082846 |
| 39 | threonic acid | 3.2696 | 0.0020431 | 2.6897 | 0.0091154 |
| 40 | 1-monoolein | 3.246 | 0.0021857 | 2.6604 | 0.0091746 |
| 41 | uric acid | 3.2416 | 0.0022137 | 2.6549 | 0.0091746 |
| 42 | oleic acid | -3.236 | 0.0022489 | 2.648 | 0.0091746 |
| 43 | ribose | 3.2332 | 0.0022673 | 2.6445 | 0.0091746 |
| 44 | cysteine | 3.165 | 0.0027498 | 2.5607 | 0.010728 |
| 45 | nicotinic acid | -3.1618 | 0.0027745 | 2.5568 | 0.010728 |
| 46 | linoleic acid | 3.116 | 0.0031546 | 2.5011 | 0.011933 |
| 47 | D-erythro-sphingosine | -3.061 | 0.0036766 | 2.4346 | 0.013611 |
| 48 | thymidine | -3.0488 | 0.0038021 | 2.42 | 0.013783 |
| 49 | pseudo uridine | 3.0166 | 0.0041551 | 2.3814 | 0.014755 |
| 50 | isothreonic acid | 2.9366 | 0.0051679 | 2.2867 | 0.017984 |

Important features selected by volcano plot with fold change (FC) threshold (x) 2 and t-tests threshold (y) 0.1. Both fold changes and p values are log transformed. The further its position away from the (0,0), the more significant the feature is.

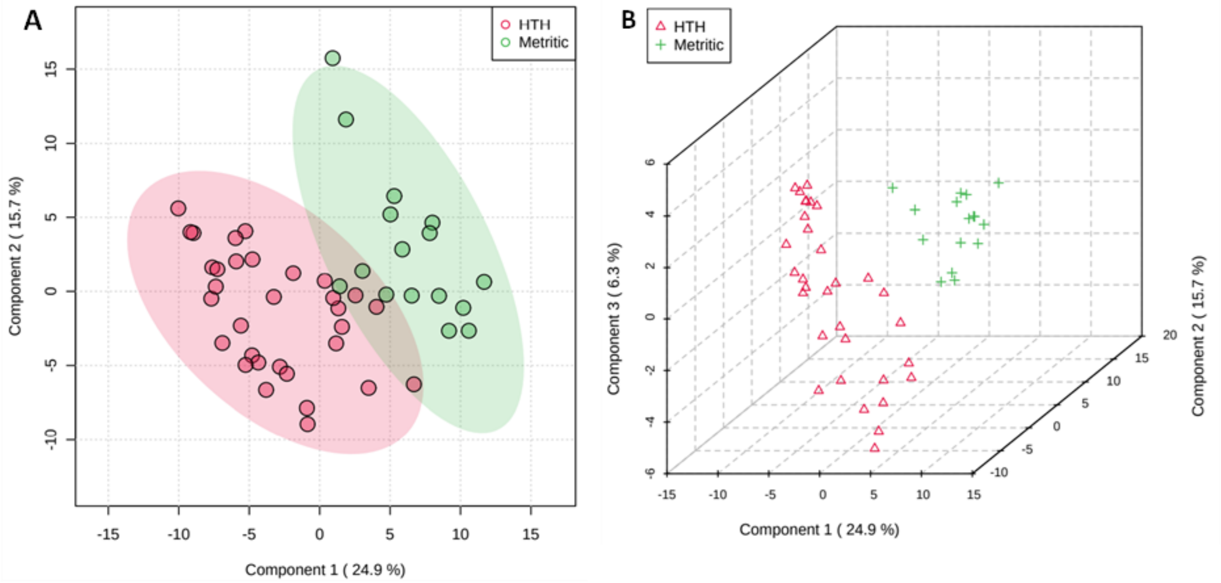


Figure 3-3. Partial least square-discriminant analysis (PLS-DA) of vaginal-uterine discharge metabolites at 7 DIM in healthy cows compared to animals developing metritis. Scores plot between the selected principal components; the explained variances are shown in brackets (A). 3-D representation of known (B) metabolome composition is displayed to demonstrate that there is a metabolome difference at 7 DIM between cows developing or not metritis.

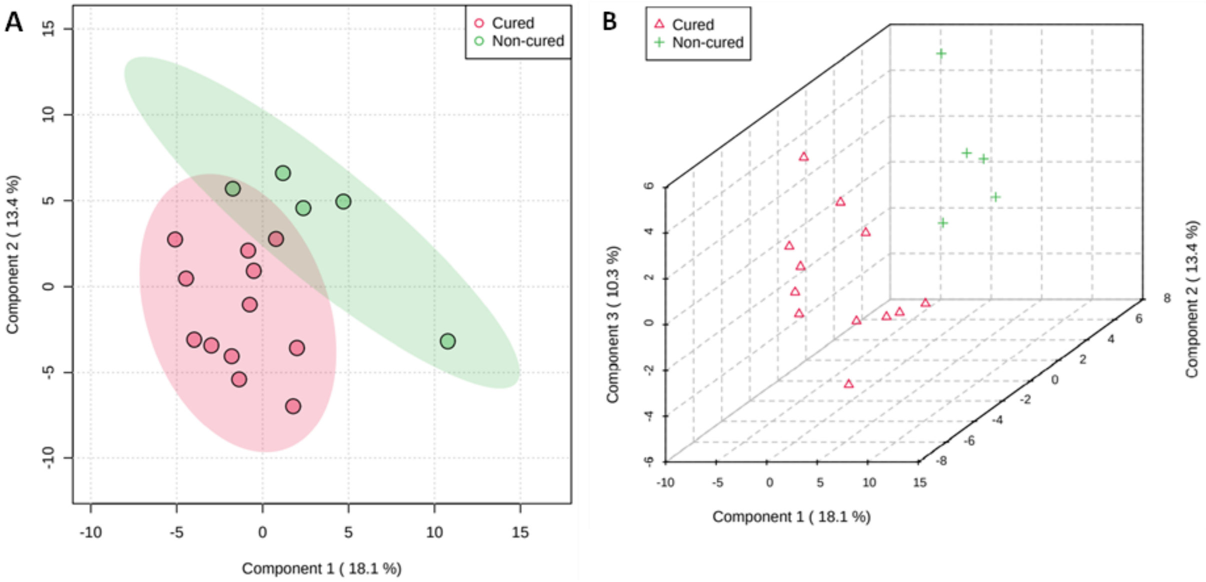


Figure 3-4. Partial least square-discriminant analysis (PLS-DA) of vaginal-uterine discharge metabolites of metritic cows 4 days after metritis diagnosis. Scores plot between the selected principal components; the explained variances are shown in brackets (A). 3-D representation of known (B) metabolome composition is displayed to demonstrate that after 4 days after metritis diagnosis there is a metabolome difference in cows curing and not from metritis.

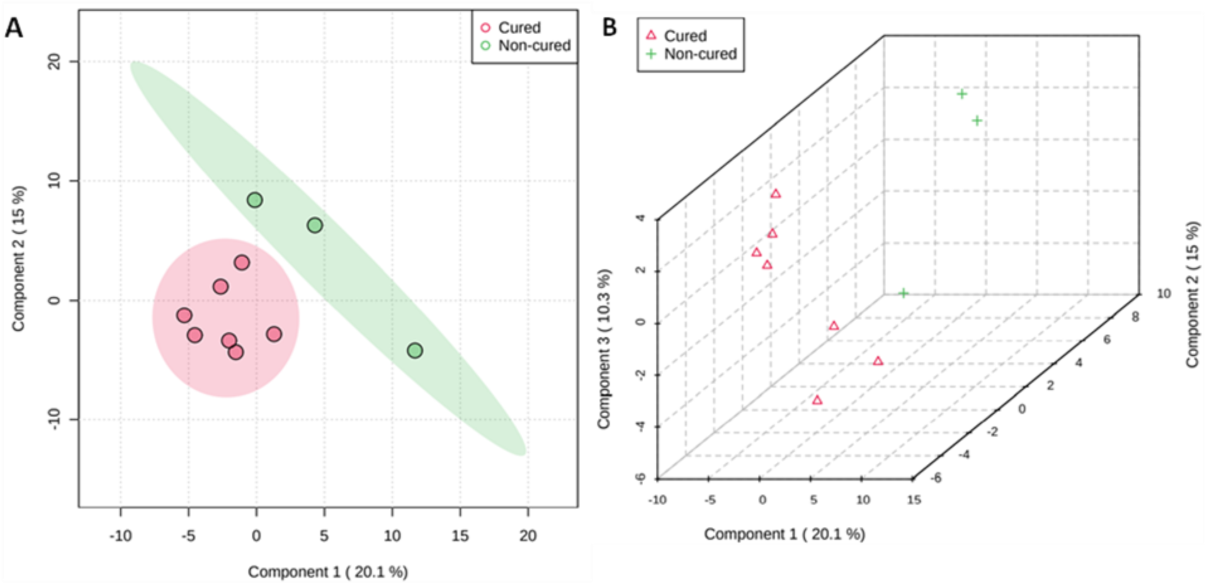


Figure 3-5. Partial least square-discriminant analysis (PLS-DA) of vaginal-uterine discharge metabolites four days after metritis diagnosis. All metritic cows received ceftiofur treatment. Scores plot between the selected principal components; the explained variances are shown in brackets (A). 3-D representation of known (B) metabolome composition is displayed to demonstrate that after 4 days after metritis diagnosis there is a metabolome difference in cows curing and not from metritis even after receiving ceftiofur treatment.

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# BIOGRAPHICAL SKETCH

Eduardo Barros de Oliveira was born in Brazil. He is the fourth generation of cattle ranchers, and he spent his childhood on his family’s farm. That was where he had the opportunity to be in contact with farm animals and decided that veterinary medicine was the best field to pursue as a career.

Eduardo attended Universidade Federal de Goiás (UFG) in Goiânia, Goiás, Brazil. He received the DVM degree in 2015. In addition to his responsibilities as student, he was the teaching assistant in a reproduction class for 2 years. After graduating in 2015, Eduardo was hired by Alta Genetics Brazil as an associate veterinarian to work in the areas of medicine, production, and reproduction.

In 2016, Eduardo moved to the United States and got involved in several research projects at the University of Florida. In 2018, he was accepted in the Master of Science program in Veterinary Medical Science at the College of Veterinary Medicine of the University of Florida, Department of Large Animal Clinical Sciences. Upon completion of his MSc program, Eduardo moved to the University of California Davis to continue his post-graduate studies in a clinical residency in dairy production medicine in the Veterinary Medicine Teaching and Research Center in Tulare, California and finished it in 2022.

In 2020, he was accepted for the Ph.D. program at the animal biology group at the University of California – Davis. In August of 2022, Eduardo accepted a job offer from Zoetis Animal Health and started to work as a Dairy Production Specialist. Eduardo sees his future closely associated with the dairy industry, especially in health, reproduction, and management. He has the strong belief that educating and generating knowledge for producers, farmworkers, practitioners, and consumers is the most important legacy that he can leaves during his career.